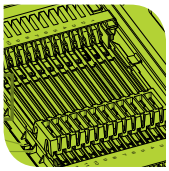
Sample Prep + 2-D Electrophoresis + Imaging



2-D Electrophoresis Workflow
How-To Guide

Fourth Edition

BIO-RAD

About This Guide

This guide describes the experimental methods and tools used in 2-D electrophoresis and proteomics research. It provides background information about technologies common to all proteomics studies as well as protocols and advice you can use as a starting point for your studies. This guide also explains how experimental conditions can be varied and interpreted to optimize your results and provides an extensive set of references that you can consult for more information. Since each sample, experimental approach, and objective is different, this guide offers ideas for developing customized protocols suitable for the analysis of your samples.

Bio-Rad's Proteomics Program

From sample preparation to protein analysis, Bio-Rad's tools provide you with choices in methodology, protocols, and products. Our informative 2-D Electrophoresis and Analysis Applications and Technologies web pages are a valuable resource with video tutorials, protocols, troubleshooting tips, and much more. To learn more, visit www.bio-rad.com/2DElectroAnalysis.

PART I

Theory and Product Selection

CHAPTER 1

Overview of Two-Dimensional Electrophoresis

The Context of Proteomics

Proteome analysis (proteomics) is the comprehensive analysis of proteins present in a sample and representing a particular physiological state at a particular point in time. The aim of proteomics is to determine the presence, relative abundance, and posttranslational modification state of a large fraction of the proteins in a sample (Wilkins et al. 1996). Since proteins are directly involved in cellular structure, regulation, and metabolism, proteomics can often yield a more informative and accurate picture of the state of a living cell than can analysis of the genome or mRNA.

One of the greatest challenges of proteome analysis is the reproducible separation of complex protein mixtures while retaining both qualitative and quantitative relationships. Many combinations of techniques can be used to separate and analyze proteins, but two-dimensional (2-D) electrophoresis is uniquely powerful in its ability to separate hundreds to thousands of products simultaneously (Choe and Lee 2000). This technique uses two different electrophoretic separations, isoelectric focusing (IEF) and SDS-PAGE, to separate proteins according to their isoelectric point (pI) and molecular weight. The identities of individual protein spots from the gel can then be identified by mass spectrometry (MS) of their tryptic peptides. Together with computer-assisted image evaluation systems for comprehensive qualitative and quantitative examination of proteomes, proteome analysis also allows cataloguing and comparison of data among groups of researchers.

Other common methods of proteome analysis involve the proteolytic digestion of sample proteins and the chromatographic separation of the resulting peptides coupled directly to mass spectrometric analysis. Peptides are identified by referencing a database, and their proteins of origin are inferred. While these methods are largely automatable and provide an impressive depth of proteome coverage, some information is lost when analyzing protein fragments instead of intact proteins. The 2-D electrophoresis approach maintains proteins in their intact states and enables the study of isoform distribution, which is not possible if the sample is proteolytically digested prior to separation. Since proteins can be selected through image analysis, mass spectrometry need be applied only to the proteins of interest. This is an important consideration when access to instrumentation or the expense of mass spectrometric analysis is a limitation.

The suitability of 2-D electrophoresis to proteome analysis is clear, but its applications also extend to biomarker detection, development of drug and other therapies, and optimization and development of protein purification strategies.

Overview of Experimental Design

The general workflow in a 2-D electrophoresis experiment (Figure 1.1) and some of the factors affecting the way the experiment is performed are outlined next.

2-D Electrophoresis Workflow

Sample Preparation

Prepare the protein at a concentration and in a solution suitable for IEF. Choose a method that maintains the native charge, solubility, and relative abundance of proteins of interest.

First-Dimension Separation: IEF

Separate proteins according to pI by IEF. Select the appropriate IPG strip length and pH gradient for the desired resolution and sample load. Select appropriate sample loading and separation conditions.

Second-Dimension Separation: SDS-PAGE

Separate proteins according to size by SDS-PAGE. Select the appropriate gel size and composition and separation conditions.

Detection

Visualize proteins using either a total protein stain or fluorescent protein tags. Select a staining technique that matches sensitivity requirements and available imaging equipment.

Image Acquisition and Analysis

Capture digital images of the 2-D protein patterns using appropriate imaging equipment and software. Then analyze the patterns using 2-D analysis software.

Protein Excision, Digestion, and Identification

Excise protein spots of interest from the gel, digest the proteins, and analyze the digests by MS.

Fig. 1.1. General workflow for a 2-D experiment.

Sample Preparation
Effective sample preparation is key for the success of the experiment. The sample dictates the type of extraction technique used, and the solubility, charge, and pI of the proteins of interest affect the method of solubilization. The protein fraction used for 2-D electrophoresis must be solubilized in a denaturing solution of low ionic strength; this solution cannot contain components that alter protein size or charge. Sample preparation also involves optional steps to deplete abundant proteins, reduce the complexity of the protein mixture, or select a subproteome of interest. Details and recommendations for sample preparation can be found in Chapter 2.

First-Dimension Separation: IEF
In 2-D electrophoresis, the first-dimension separation step is IEF. Proteins are separated electrophoretically on the basis of their pI, the pH at which a protein carries no net charge. For general proteome analysis, IEF is best performed in an immobilized pH gradient (IPG) strip and under conditions aimed at completely denaturing and solubilizing all the proteins in the sample (as opposed to native IEF, which aims to preserve native structures and activities). Chapter 3 discusses IEF.

Second-Dimension Separation: SDS-PAGE
The second-dimension separation step is SDS-PAGE, where the proteins already separated by IEF are further separated by their size. Prior to second-dimension separation, an equilibration step is applied to the IPG strip containing the separated proteins. This process reduces any disulfide bonds that may have re-formed during the first dimension and alkylates the resultant sulfhydryl groups. Concurrently, the proteins are complexed with SDS for separation on the basis of size. Following electrophoretic separation on a slab gel, the result is a two-dimensional array of separated protein "spots" (Figure 1.2). Second-dimension SDS-PAGE is discussed in Chapter 4.

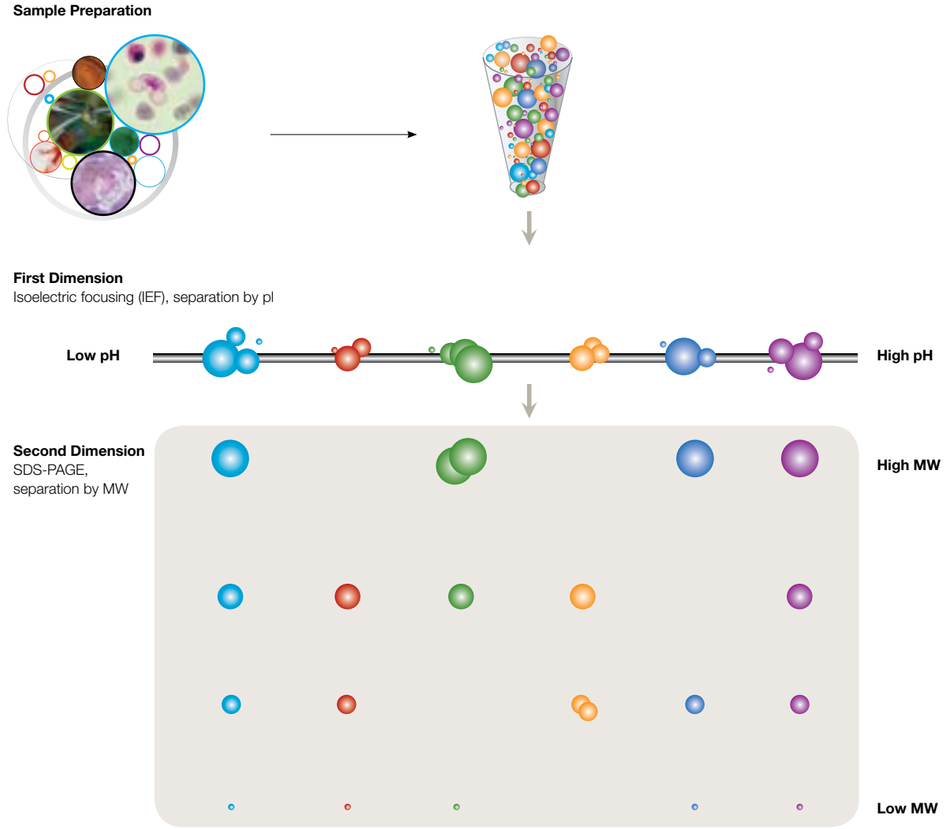


Fig. 1.2. 2-D electrophoresis. Protein spots result from two separations: first by pI (IEF) and then by size (SDS-PAGE).

Detection
Proteins separated in gels are usually not visible to the naked eye and must, therefore, be either stained or labeled for visualization. Several factors determine the best choice of staining method, including desired sensitivity, linear range, ease of use, expense, and the type of imaging equipment available. There is no ideal universal stain. Sometimes proteins are detected after transfer to a membrane support by western blotting. These topics are discussed in Chapters 5 and 6.

Image Acquisition, Analysis, and Spot Cutting
The ability to collect data in digital form is one of the major factors that make 2-D gels a practical means of collecting proteome information. It allows the unbiased comparison of samples and gels, transfer of information among research groups, and cataloguing of data. Many types of imaging devices interface with software designed specifically to collect, interpret, and compare proteomics data.

Once interesting proteins are selected by differential analysis or other criteria, the proteins can be excised from gels and identified by mass spectrometry. The ExQuest™ spot cutter, which can be operated independently or programmed to run from PDQuest™ software, automatically cuts selected protein spots from gels with precision and deposits them into the wells of microplates.

Imaging equipment, software, and the ExQuest spot cutter are discussed in Chapter 6.

Protein Digestion and Identification by Mass Spectrometry
The excised gel plugs are destained and enzymatically digested (usually with trypsin) in preparation for identification by mass spectrometry. The use of mass spectrometry for precise mass and partial sequence determination, coupled with the availability of protein sequence databases, has made high-throughput protein identification possible. An overview of this process is provided in Chapter 7.

CHAPTER 2

Sample Preparation

The Importance of Sample Preparation

Sample preparation contributes significantly to the overall reproducibility and accuracy of protein expression analysis (Link 1999, Rabilloud 1999, Molloy 2000). Without proper sample preparation, proteins may not separate from one another or may not be represented in the 2-D pattern.

A successful sample preparation strategy enhances separation quality by:

- Effectively and reproducibly solubilizing proteins of interest
- Preventing protein aggregation and loss of solubility during IEF
- Preventing proteolysis or other chemical or enzymatic protein modifications
- Removing or minimizing the effect of contaminants such as salts, detergents, nucleic acids, and other interfering molecules
- Yielding proteins of interest at detectable levels, which may require fractionation to reduce protein sample complexity or removal of interfering abundant or irrelevant proteins

This chapter provides an overview of the principles and recent developments in sample preparation strategies prior to first-dimension IEF.

General Considerations

Since protein types and sample origins show great diversity, there is no universal sample preparation method. In addition, some proteins simply cannot be solubilized under conditions compatible with IEF. Sample preparation procedures must be optimized empirically and tailored to each sample type and experimental goal. The following general sample preparation guidelines should be kept in mind:

- Keep the sample preparation workflow as simple as possible; increasing the number of sample handling steps may increase variability and the risk of sample loss
- With cell or tissue lysates, include protease inhibitors to minimize artifacts generated by proteolysis; protease inhibitors are generally not required for samples like serum or plasma
- Solubilize proteins in a solution that is compatible with IEF. Incubate proteins in 2-D lysis solution for at least 30 min at room temperature (denaturation, solubilization, and disaggregation are time-dependent processes)
- Determine the amount of total protein in each sample using a protein assay that is compatible with chemicals in your samples
- Avoid freeze-thaw cycles; use protein extracts immediately or aliquot them into appropriately sized batches and store them at –70°C

Cell Lysis

The effectiveness of a cell lysis method determines the accessibility of intracellular proteins for extraction and solubilization. Different biological materials require different lysis strategies, which can be divided into two main categories: gentle methods and harsher methods (Table 2.1).

- Use gentle cell disruption protocols with cells that lyse easily, such as blood cells and tissue culture cells
- Use harsher methods, which are based mainly on mechanical rupture (Goldberg 2008), with biological materials that have tough cell walls (for example, plant cells and tissues, and some microbes)
- When working with a new sample, compare at least two different cell disruption protocols with respect to yield (by protein assay) and qualitative protein content (by one-dimensional SDS-PAGE)
- Optimize the power settings of mechanical rupture systems and the incubation times of lysis approaches
- Mechanical cell lysis usually generates heat; use cooling where required to avoid overheating the sample

A number of other components are often added to disruption protocols. Sand, resin, or glass beads facilitate the disruption of tissues and of plant and yeast cell walls when added to manual grinding procedures. Hypotonic buffers cause cells to burst more readily under physical shearing, and enzymes such as cellulase, pectinase, lyticase, and lysozyme are added to break down plant, yeast, and bacterial cell walls. Nucleases can be added to remove nucleic acids, which can increase sample viscosity and interfere with subsequent separation (see the Removal of Interfering Substances section).

Table 2.1. Suitability of cell disruption methods for various sample types.

Technique	Description	Bacteria	Yeast, Algae, Fungi	Seeds	Green Plant Material	Soft Tissues	Mammalian Cell Culture
Gentle Methods							
Osmotic lysis	Suspension of cells in hypotonic solution; cells swell and burst, releasing cellular contents	–	–	–	–	–	•
Freeze-thaw lysis	Freezing of cells in liquid nitrogen and subsequent thawing	–	–	–	–	–	•
Detergent lysis	Suspension of cells in detergent-containing solution to solubilize the cell membrane; this method is usually followed by another disruption method, such as sonication	–	–	–	–	–	•
Enzymatic lysis	Suspension of cells in iso-osmotic solutions containing enzymes that digest the cell wall (for example, cellulase and pectinase for plant cells, lyticase for yeast cells, and lysozyme for bacterial cells); this method is usually followed by another disruption method, such as sonication	•	•	–	•	–	–
Harsher Methods							
Sonication	Disruption of a cell suspension, cooled on ice to avoid heating and subjected to short bursts of ultrasonic waves	•	•	–	–	–	•
French press	Application of shear forces by forcing a cell suspension through a small orifice at high pressure	•	•	–	•	–	•
Grinding	Breaking cells of solid tissues and microorganisms with a mortar and pestle; usually, the mortar is chilled with liquid nitrogen and the tissue or cells are ground to a fine powder	•	•	•	•	•	–
Mechanical homogenization	Homogenization with either a handheld device (for example, Dounce and Potter-Elvehjem homogenizers), blenders, or other motorized devices; this approach is best suited for soft, solid tissues	–	–	–	•	•	–
Glass-bead homogenization	Application of gentle abrasion by vortexing cells with glass beads	•	•	–	–	–	•

All but the most gentle cell disruption methods destroy the compartmentalization of a cell, causing the release of hydrolases (phosphatases, glycosidases, and proteases). These enzymes modify proteins in the lysate, which complicates differential analysis. The data generated by 2-D electrophoresis are only meaningful when the integrity of the sample proteins reflects the state in which they are found in the living organism. Avoid enzymatic degradation by using one or a combination of the following techniques:

- Disrupt the sample or place freshly lysed samples in solutions containing strong denaturing agents such as 7–9 M urea, 2 M thiourea, or 2% SDS. In this environment, enzymatic activity is often negligible
- Perform cell lysis at low temperatures to diminish enzymatic activity
- Lyse samples at pH >9 by adding a base such as sodium carbonate or Tris(hydroxymethyl)-aminomethane (Tris) to the lysis solution (proteases are often least active at basic pH)
- Add protease inhibitors to the lysis solution. Examples include either small molecules, such as phenylmethylsulfonyl fluoride (PMSF), aminoethyl-benzene sulphonyl fluoride (AEBSF), tosyl lysine chloromethyl ketone (TLCK), tosyl phenyl chloromethyl ketone (TPCK), ethylenediaminetetraacetic acid (EDTA), and benzamidine, or peptide protease inhibitors such as leupeptin, pepstatin, aprotinin, and bestatin. For best results, use a combination of inhibitors in a protease inhibitor cocktail
- If protein phosphorylation is to be studied, include phosphatase inhibitors such as fluoride or vanadate

Following cell disruption:

- Check the efficacy of cell disruption by light microscopy (if the sample is a cell suspension)
- Centrifuge all extracts extensively (20,000 × g for 15 min at 15°C) to remove any insoluble material; solid particles may block the pores of the IPG strip

Products for Cell Lysis and Protein Extraction

ReadyPrep™ mini grinders contain a grinding tube, grinding resin, and fitted pestle and offer an easy, efficient mechanism for manually grinding small biological samples. The grinding resin is a neutral abrasive material made of a high-tensile microparticle that does not bind protein or nucleic acids. The combination of the pestle and resin effectively disrupts animal or plant tissues and cells. ReadyPrep mini grinders are available for purchase separately or as components of the MicroRotofor™ cell lysis kits.

Bio-Rad also offers a range of kits for cell disruption and protein extraction:

- MicroRotofor lysis kits[1] provide convenient, effective methods optimized for the preparation of protein samples from mammalian, plant, yeast, and bacterial sources. Depending on the sample type, these kits employ tissue maceration using ReadyPrep mini grinders and/or solubilization into a chaotropic extraction buffer
- The ReadyPrep protein extraction kit (total protein) uses the powerful zwitterionic detergent ASB-14 in a strongly chaotropic solubilization buffer to prepare total cellular protein extracts suitable for 2-D electrophoresis (depending on sample type, additional cell lysis protocols may be needed when using this kit)
- Other ReadyPrep protein extraction kits facilitate extraction of specific classes of proteins and are discussed later in this chapter

Such standardized lysis and extraction protocols are often useful for initial proteomic analyses and for consistent sample preparation.

ReadyPrep Mini Grinder

MicroRotofor Lysis Kit

ReadyPrep Protein Extraction Kit

Products for cell lysis and protein extraction. A number of other ReadyPrep protein extraction kits facilitate disruption and extraction of specific classes of proteins.

[1] For added convenience, the extraction buffer included with these kits can also be used as the sample solution for IEF with the MicroRotofor cell or with IPG strips.

Protein Solubilization

Proteins in a biological sample are often associated with other proteins, integrated into membranes, or parts of large complexes. Protein solubilization is the process of breaking interactions involved in protein aggregation (Rabilloud 1996), which include disulfide and hydrogen bonds, van der Waals forces, and ionic and hydrophobic interactions. If these interactions are not disrupted, proteins can aggregate or precipitate, resulting in artifacts or sample loss. For successful 2-D electrophoresis, proteins must be well solubilized.

Sample lysis solutions typically contain a number of compounds that meet the requirements, both electrically and chemically, for compatibility with IEF. To allow high voltages to be applied during IEF without producing high currents, the compounds must not increase the ionic strength of the solution. In some cases, it may be necessary to prepare samples using additives that facilitate protein solubilization but that have limited compatibility with IEF (for example, salts and SDS). In these cases, the potentially interfering substance must be removed prior to sample application, or actions must be taken to mitigate its effect (see the Removal of Interfering Substances section). See Chapter 9 for sample preparation procedures and solutions; for a thorough discussion of solubilization methods, refer to Rabilloud (2000).

Chaotropic Agents

These compounds disrupt hydrogen bonds and hydrophobic interactions both between and within proteins. When used at high concentrations, chaotropic agents disrupt secondary protein structure and bring into solution proteins that are otherwise insoluble. The neutral chaotropic agent urea is used at 5–9 M, often with up to 2 M thiourea, which can dramatically increase the number of proteins solubilized (Rabilloud et al. 1997). Thiourea is weakly soluble in water but more soluble in high concentrations of urea; therefore, a mixture of 2 M thiourea and 5–8 M urea is used when strongly chaotropic conditions are required. Charged chaotropic agents such as guanidine hydrochloride are incompatible with IEF.

If using thiourea during sample preparation, also add it to the first-dimension rehydration solution; otherwise, the proteins that require thiourea for solubility will come out of solution during IEF.

Urea and thiourea can hydrolyze to cyanate and thiocyanate, respectively; these products modify amino groups on proteins (carbamylation) and give rise to artifactual charge heterogeneity. Since heat promotes this hydrolytic reaction, never heat urea- or thiourea-containing solutions above 37°C in the presence of protein (McCarthy et al. 2003).

Detergents

Detergents disrupt hydrophobic interactions between and within proteins and are classified as neutral, zwitterionic, anionic, and cationic (Luche et al. 2003). Some proteins, especially membrane proteins, require detergents for solubilization during isolation and for maintaining solubility during IEF.

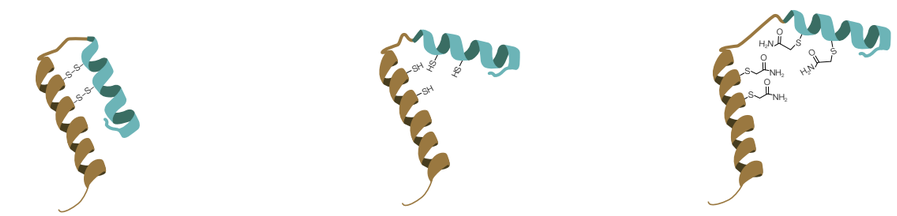
Sample preparation for 2-D electrophoresis commonly uses neutral or zwitterionic (having both positive and negative charges resulting in a neutral net charge) detergents at concentrations of 1–4%, since these detergents do not introduce a net charge and therefore allow proteins to migrate at their own charges during IEF. Examples of neutral detergents include Tween, octylglucoside, dodecyl maltoside, Triton X-100, and Triton X-114. Examples of zwitterionic detergents include CHAPS, CHAPSO, ASB-14, and SB 3-10. In practice, only a few detergents are used in IEF (Table 2.2). With few exceptions, only a single detergent should be used because the effects of detergents are not additive and can be unpredictable in combination. Anionic and cationic detergents are generally not suitable for IEF.

SDS is unparalleled in its ability to efficiently and rapidly solubilize proteins. Although SDS is incompatible with IEF as an anionic detergent, it can be used in the initial preparation of concentrated protein samples. In these cases, another IEF-compatible detergent must be used in excess to disrupt the binding of SDS to protein (Ames and Nikaido 1976). Also to be considered is how the detergent interacts with high concentrations of urea. When using SB 3-10, for example, the urea concentration is limited to 5 M, but ASB-14 can be used with 9 M urea (Chevallet et al. 1998).

Reducing Agents

Reducing agents cleave disulfide bond cross-links within and between protein subunits, thereby promoting protein unfolding and maintaining proteins in their fully reduced states. The compounds used for 2-D sample preparation are either sulfhydryl or phosphine reducing agents. Examples of sulfhydryl reductants include dithiothreitol (DTT), dithioerythritol (DTE), and β-mercaptoethanol (BME). DTT and DTE can be used at lower concentrations than β-mercaptoethanol and are more commonly used, but high concentrations of DTT can affect the pH gradient since its pKa is around 8. Examples of phosphine reductants include tributylphosphine (TBP) and Tris-carboxyethylphosphine (TCEP). These reducing agents can be used at lower concentrations and over a wider pH range than the sulfhydryl reductants; however, their use is limited by low solubility and instability (TBP) or a highly charged characteristic (TCEP).

Reducing agents added during protein extraction help to solubilize proteins; during IEF, however, reducing agents such as DTT become depleted from the basic end of pH gradients extending above pH 8, which can cause proteins to aggregate and precipitate (Hoving et al. 2002). The result is streaking and other random spot patterns, particularly in the alkaline regions of the IPG strip (Herbert et al. 2001). To address this problem, proteins can be reduced with TBP and then irreversibly alkylated with iodoacetamide (Figure 2.1). This treatment blocks protein sulfhydryls and prevents proteins from aggregating and precipitating due to oxidative cross-linking, ensuring that proteins remain soluble throughout electrophoresis (Figure 2.2).



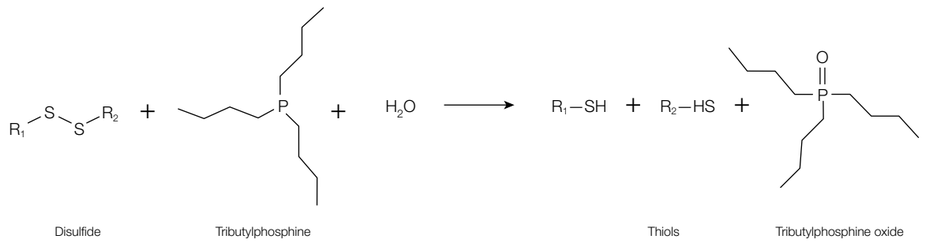


Fig. 2.1. Reduction and alkylation.

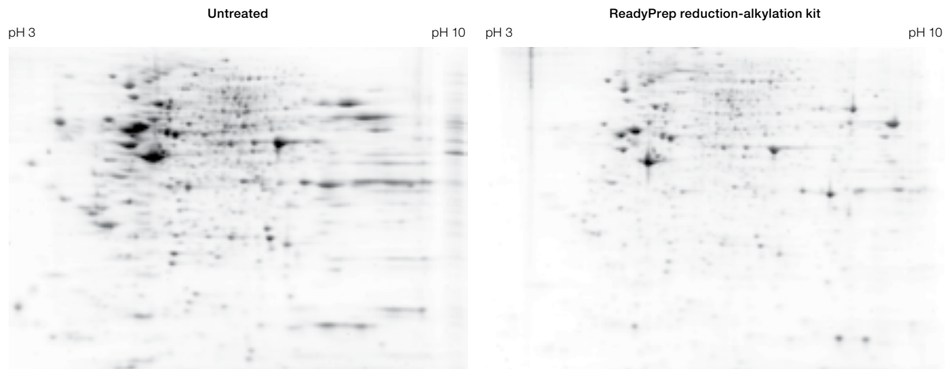


Fig. 2.2. Effect of treatment with the ReadyPrep reduction-alkylation kit. Human HeLa cell extract (100 µg) separated by 2-D electrophoresis (first dimension on 11 cm ReadyStrip™ IPG strips pH 3–10, second dimension using 12% Criterion™ gels) and stained with Flamingo™ protein gel stain. The sample treated with the ReadyPrep reduction alkylation kit (right) and shows much better spot resolution than the untreated sample (left), especially in the basic range of the gel.

ReadyPrep Reduction-Alkylation Kit

Bio-Rad's ReadyPrep reduction-alkylation kit provides the reagents for reduction and alkylation of sample proteins prior to IEF. Its use produces a 2-D pattern with more spots, fewer streaks, and greater reproducibility.

ReadyPrep Reduction-Alkylation Kit

Ampholytes, Buffers, and Other Additives

Sample solution components that modify pH or impart ionic strength affect the solubilization of proteins during sample preparation and strongly influence 2-D electrophoresis.

Carrier ampholyte mixtures increase both buffering power and ionic strength. Unlike non-ampholytic ions, they do not interfere with IEF and can, in fact, improve protein solubility by "salting in" proteins that are otherwise insoluble under IEF conditions. In addition, carrier ampholytes can diminish protein-matrix interactions, which tend to occur at the basic end of an IPG strip and lead to streaking caused by precipitation (Righetti and Gianazza 1987). Carrier ampholytes are routinely added to solutions used during IEF with IPG strips and can be of value during protein extraction as well.

Since proteins are often more soluble and proteases are less active at higher pH, a base such as Tris may be included in a lysis solution to elevate pH.

Many proteins also require ions in solution for optimum solubility. Normally, this is achieved by adding salt to the sample solution; however, adding salt prior to IEF increases conductivity and consequently limits the voltage at which IEF can be performed until the salt is eventually removed from the system. Ions also leave the IPG strip during IEF, causing any protein requiring ions for solubility to precipitate. Proteins also become less soluble as they approach their pI; they may precipitate at their pI in a phenomenon known as isoelectric precipitation or pI fallout.

Table 2.2. Summary of compounds used in 2-D electrophoresis sample solutions. Refer to Ordering Information (Appendix C) for catalog numbers and details of options available for purchase.

Compound or product	Role in Solution	Concentration Range	Comments
Urea	Chaotrope	5–9.5 M	Present during first-dimension IEF
Thiourea	Chaotrope	2 M	Used with urea, usually in the combination 7 M urea, 2 M thiourea; more effective than urea alone for solubilizing hydrophobic or high molecular weight proteins
CHAPS	Detergent	1–4% (w/v)	Zwitterionic detergent that may enhance protein solubility with minimal disruptive effect on 2-D electrophoresis (Perdew et al. 1983)
CHAPSO	Detergent	1–4% (w/v)	Zwitterionic detergent similar to CHAPS
NP-40	Detergent	0.5–1% (w/v)	Neutral detergent originally used in 2-D electrophoresis (O'Farrell 1975, Görg et al. 1988); its use has been largely superseded by CHAPS (Görg et al. 2004)
Triton X-100	Detergent	0.5–1% (w/v)	Neutral detergent similar to NP-40 also used for 2-D sample preparation (Kawaguchi and Kuramitsu 1995)
SB 3-10	Detergent	1–2% (w/v)	Zwitterionic detergent shown in some cases to give better solubilization than CHAPS; insoluble in higher concentrations of urea and generally used with 5 M urea, 2 M thiourea (Rabilloud et al. 1997)
ASB-14	Detergent	1–2% (w/v)	Zwitterionic detergent developed for solubilization of membrane proteins to be analyzed by 2-D electrophoresis (Chevallet et al. 1998)
ASB-C8Ø	Detergent	1–2% (w/v)	Zwitterionic detergent developed for solubilization of membrane proteins to be analyzed by 2-D electrophoresis (Chevallet et al. 1998)
Sodium dodecyl sulfate (SDS)	Detergent	Up to 2% (w/v) during sample preparation, no more than 0.2% (w/v) during IEF	Anionic detergent widely used in sample preparation for electrophoresis and unparalleled in its ability to solubilize protein; also effective at inactivating proteases and other undesirable enzymatic activities. It is, however, incompatible with IEF unless diluted to 0.2% or less and used with at least an eightfold excess of an IEF-compatible detergent such as CHAPS
Dithiothreitol (DTT)	Reductant	20–60 mM	Most commonly used sulfhydryl reductant for 2-D electrophoresis
β-Mercaptoethanol	Reductant	1–5% (v/v)	Sulfhydryl reductant originally used for 2-D electrophoresis (O'Farrell 1975); must be used at a relatively high concentration and can cause disturbances to IEF, so is rarely used
Tributylphosphine (TBP)	Reductant	2 mM	Phosphine reductant effective at low concentrations and reported to enhance solubilization of recalcitrant samples (Herbert et al. 1998). It has low water solubility and is unstable and therefore not recommended as the sole reductant for first-dimension IEF
Tris-carboxyethylphosphine (TCEP)	Reductant	2–40 mM	Phosphine reductant that may be useful during sample preparation; it is highly charged and so is not recommended as the sole reductant present during first-dimension IEF
Tris	Base	10–40 mM	(Unbuffered) free base often added to sample preparation solutions to raise the pH to a range where proteolysis is minimal and proteins are optimally soluble. Other bases (for example, potassium carbonate or spermine) are occasionally used as well (Rabilloud 1999). If Tris is used during sample preparation, it should be diluted to 20 mM or less for first-dimension IEF, as it may cause disturbances in the basic pH range
Bio-Lyte® ampholytes	Carrier ampholyte	0.2–1.0% (w/v)	Carrier ampholytes may be used during sample preparation to enhance protein solubility. Although IEF with IPG strips does not require carrier ampholytes for pH gradient generation, the presence of a relatively low (0.2% [w/v]) concentration of carrier ampholyte is essential for optimum resolution. Use pH 3–10 ampholytes or ampholytes appropriate to the IPG strip pH range

Removal of Interfering Substances

Impurities such as ionic detergents, lipids, nucleic acids, salts and other ionic compounds, and even high-abundance proteins can impact a 2-D electrophoresis experiment by interfering with protein separation or by obscuring proteins of interest. These interfering substances can be endogenous (for example, phenolics, lipids, and nucleic acids) or exogenous (added during sample preparation; for example, salts and detergents). Either way, removing these impurities prior to analysis or mitigating their effect is often essential for good results.

General Considerations

Though removal or mitigation of interfering substances often yields clearer 2-D patterns and improves resolution of protein spots, any treatment of the sample can reduce yield and alter the relative abundance of sample proteins. Procedures for the removal of interfering substances represent a compromise between removal of non-protein contaminants and minimal interference with the integrity and relative abundance of the sample proteins. Since proteomics aims to study the relationship among proteins in their natural state, it is important to remove an interfering substance only when necessary and by using techniques appropriate for the sample.

Protein precipitation is a common general method for contaminant removal. Conditions are chosen under which sample proteins are selectively precipitated while leaving soluble the major non-protein contaminants. Following centrifugation, the precipitated proteins are resuspended in a solution suitable for IEF. Methods used in sample preparation for 2-D electrophoresis include precipitation with TCA and acetone (Damerval et al. 1986, Görg et al. 1988) and precipitation with methanol and chloroform (Wessel and Flügge 1984). Precipitation procedures also have the benefit of concentrating sample protein, which is often necessary for effective sample application.

Individual types of interfering contaminants cause specific problems and can be removed or mitigated in different ways. The most prevalent interfering contaminants and their removal methods are discussed next.

Nucleic Acids (DNA and RNA)

Nucleic acids, particularly DNA, can interfere with IEF (for example by clogging gel pores) and increase sample viscosity, thus limiting the effectiveness of cell lysis and sample application. Because smaller nucleic acids are generally tolerated better, strategies to reduce nucleic acid interference involve either shearing or enzymatic digestion: sonication shears DNA and renders the sample less viscous, and addition of nuclease digests nucleic acids to oligo- or mononucleotides.

Nucleases are often employed during sample preparation, particularly with bacterial lysates in which nucleic acid:protein ratios are high. Successful application of nuclease treatment requires attention to three factors:

- Nucleases may be inactive under the strongly denaturing conditions often used to prepare protein samples for 2-D electrophoresis
- DNase requires magnesium ions for activity
- Nucleases are proteins and can appear in the 2-D pattern as extra spots

Benzonase is a nuclease with properties that make it particularly useful in sample preparation for 2-D electrophoresis (Chan et al. 2002). It is active in the presence of urea, and the amount required for treatment is usually not visible in a 2-D gel. It is applied in the presence of 1 mM $MgSO_4$ or $MgCl_2$. The magnesium ions are subsequently sequestered with EDTA in order to inhibit proteases that may require metal ions for activity.

Polysaccharides

Polysaccharides can interfere with electrophoresis by clogging gel pores and by forming complexes with proteins. Like nucleic acids, they can also cause a sample to be viscous, making it difficult to work with. Polysaccharides are a particularly prominent problem with plant-derived samples.

Centrifugation may be used to remove high molecular weight polysaccharides. Phenol extraction, followed by precipitation with ammonium acetate in methanol, is a commonly used method that is very effective at removing polysaccharides in plant samples (Hurkman and Tanaka 1986, Wang et al. 2008).

Phenolic Compounds

Phenolic compounds are found in all plants and in some microorganisms and they can modify proteins in an enzyme-catalyzed oxidative reaction. The modification can cross-link proteins together or render them insoluble. The reaction can be prevented with reductants such as DTT, β-mercaptoethanol, or ascorbic acid, and the enzyme is inactivated by thiourea. Phenolic compounds may also be removed from the extract using the ReadyPrep 2-D cleanup kit (see the Products for Contaminant Removal sidebar) or by including polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP) in the extraction solution. These compounds bind phenolic compounds, and the precipitated complex can be removed from the extract by centrifugation (Toth and Pavia 2001). The phenol extraction procedure described above (see Polysaccharides) is also effective at removing phenolic contaminants (Hurkman and Tanaka 1986, Wang et al. 2008).

Lipids

Lipids can form insoluble complexes with proteins, but lipids can also complex with detergents, thereby reducing the detergents' effectiveness at solublilizing protein. The effect of lipids can be minimized by using excess detergent (for example, 4% CHAPS in the lysis solution when preparing lipid-rich tissues such as brain). Precipitation methods that employ organic solvents (Damerval et al. 1986, Görg et al. 1988, Wessel and Flügge 1984) or the ReadyPrep 2-D cleanup kit can also be used to remove lipids.

Salts and Other Small Ionic Compounds

IEF requires samples that are free of salts and other small ionic compounds that may interfere with pH gradient formation. Salts formed from strong acids and strong bases (for example, NaCl) dissociate into their component base and acid, which is eventually drawn to either end of the IPG strip. Until this occurs, the conductivity of the IPG strip remains high and the voltage attained is low. The flow of ions from the IPG strip is accompanied by water flow, and one end of the strip may dry out, breaking electrical contact. Weak acids and weak bases (for example, acetate, Tris, or ammonium ions) may not completely leave the IPG strip during focusing. These compounds interfere with the pH gradient, resulting in streaking and loss of resolution at one end of the pH range or the other (Figure 2.3). Amphoteric buffers such as HEPES can focus within the pH gradient, resulting in a portion of the pH gradient where proteins focus poorly.

Before

After

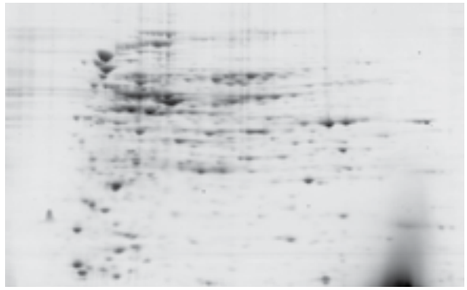


Fig. 2.3. Effect of salt removal. *E. coli* extracts containing 1 M NaCl were separated by 2-D electrophoresis before and after treatment with the ReadyPrep 2-D cleanup kit. The samples were focused using 11 cm ReadyStrip pH 3–10 IPG strips and then separated on Criterion 8–16% Tris-HCl precast gels.

Samples of low ionic strength are desired, yet many samples contain salts and small ionic compounds that are either intrinsic to the sample type or have been introduced during sample preparation. Precipitation and dialysis methods are very effective at removing ionic contaminants, as is treatment with a desalting column (Chan et al. 2002).

Prevention of Keratin Contamination

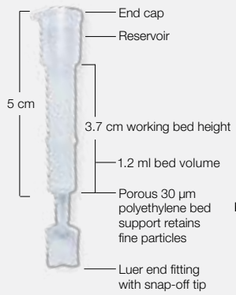
Skin keratin is a common contaminant of 2-D gels and mass spectra. It may appear in silver-stained and fluorescently stained 2-D gels as an artifact focusing near pH 5 in the 50–70 kD region, or as an irregular but distinctive vertical streaking parallel to the SDS-PAGE direction of migration. The best remedy for this keratin artifact is to avoid introducing it into the sample in the first place. Filter all monomer solutions, stock sample buffers, gel buffers, and electrode buffers through nitrocellulose and store them in sealed containers; then, clean the electrophoresis cell thoroughly with detergent. Above all, careful sample handling is important when sensitive detection methods are used, and gloves should be worn while handling samples, solution, or equipment.

Products for Contaminant Removal

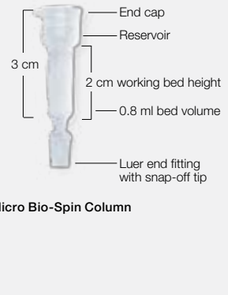
For quick and effective contaminant removal, Bio-Rad offers:

- ReadyPrep 2-D cleanup kit, which uses an optimized version of a TCA-sodium deoxycholate coprecipitation procedure (Arnold and Ulbrich-Hoffmann 1999) to quantitatively precipitate proteins while removing most interfering substances. The protein precipitation process also enables concentration of proteins from samples that are too dilute, allowing for higher protein loads that can improve spot detection
- Bio-Spin® 6 and Micro Bio-Spin™ 6 columns are ready to use and are filled with Bio-Gel® P-6 support for the quick desalting and buffer exchange of protein samples

ReadyPrep 2-D Cleanup Kit



Bio-Spin Column



Micro Bio-Spin Column

Prefractionation

Proteomic analysis is often applied to samples that have undergone prior fractionation (prefractionation), and the reasons for this are varied. In cases where only a defined subset of the proteome is under study, prefractionation can increase the chances of meaningful discovery by removing proteins not likely to be of interest from the sample. For example, in studies of mitochondrial processes, it is sensible to perform the proteomic analysis on a subcellular fraction enriched in mitochondria. In other cases, specific proteins of interest may be enriched through fractionation and analyzed by 2-D electrophoresis in the absence of potentially interfering proteins. Prefractionation can also be used to separate a sample into multiple fractions of lower complexity that can then be analyzed separately; this can enable identification of lower-abundance proteins that might otherwise be undetectable in the unfractionated sample.

Prefractionation increases the depth of proteome analysis, but it does so at the expense of a greater workload and reduced throughput. Try to use a fractionation method that generates minimal protein overlap between fractions.

Proteins can be fractionated by a number of different techniques. The choice of method depends on the sample, experimental goals, and available instrumentation:

- **Chemical and centrifugal methods** — use of selective precipitation or selective extraction or centrifugation steps to separate proteins or partition different subcellular compartments. In many instances, protein extraction protocols can incorporate fractionation steps through the selective use of certain chemical reagents
- **Electrophoretic methods** — application of liquid-phase IEF or preparative SDS-PAGE with the goal of protein enrichment. Though neither of these techniques is orthogonal to either of the two dimensions employed in 2-D electrophoresis and neither offers additional resolving power to the analysis, electrophoresis has proven useful in allowing the enrichment of low-abundance proteins. A protein in a size- or pI-enriched fraction can be subjected to 2-D electrophoresis at a higher amount relative to the unfractionated sample, allowing the analysis of proteins present below detection levels (Zuo and Speicher 2000, Fountoulakis and Juranville 2003)
- **Chromatographic methods** — use of chromatographic separation principles to enrich low-abundance proteins or generate fractions of reduced complexity (Fountoulakis et al. 1997, Badock et al. 2001, Butt et al. 2001, Smith et al. 2004, Qin et al. 2005, Yuan and Desiderio 2005). Virtually any chromatographic procedure can be used as a prefractionation step; examples include size exclusion, affinity, ion exchange, and reverse-phase resins

Using these methods alone or in combination, proteins can be separated upstream of 2-D electrophoresis (prefractionated) by their physical or chemical properties, as described below. Some of these methods, however, may introduce ionic or other contaminants that must be removed before IEF. Also, increasing the number of sample handling steps may increase variability and the risk of sample loss.

Fractionation by Subcellular Location

There are many techniques for preparing fractions enriched in subcellular organelles or membrane types, and there are several examples in which these techniques have been used to prepare samples for 2-D electrophoresis and other proteomic analyses (Huber et al. 2003). Methods for organellar fractionation generally involve differential and density gradient centrifugation (Stasyk et al. 2007, Fialka et al. 1997). However, fractionation schemes involving aqueous polymer phase separation (Tang et al. 2008) and free-flow electrophoresis (Zischka et al. 2003, Eubel et al. 2008) have been described for this purpose as well. These methods are usually specific for the source material (cells or tissue). In some cases, fractions representing different subcellular sites can be generated on the basis of solubility under different conditions (see the Fractionation by Solubility/Hydrophobicity section). These methods are more general in application.

Bio-Rad offers several ReadyPrep protein extraction kits for the isolation of fractions enriched in integral membrane and transmembrane proteins (Figure 2.4), as well as nuclear and cytoplasmic proteins (see the Products for Fractionation by Subcellular Location sidebar).

Products for Fractionation by Subcellular Location

Each of the following kits produces a fraction with a distinct protein composition:

- ReadyPrep protein extraction kit (signal) takes advantage of the limited solubility of plasma membrane microdomain structures (for example, lipid rafts and caveolae) in nonionic detergents at 4°C to yield a protein pellet that is enriched in membrane-associated signalling proteins, including glycophosphatidylinositol (GPI)-anchored proteins, caveolin and associated proteins, acetylated tyrosine kinases, and G proteins (Simons and Ikonen 1997)
- ReadyPrep protein extraction kits (membrane I and membrane II) use different techniques to isolate integral membrane and membrane-associated proteins without the need for density gradients. The membrane I kit is based on temperature-dependent partitioning of hydrophobic proteins into the detergent-rich phase of a Triton X-114/water two-phase system (Bordier 1981, Prime et al. 2000, Santoni et al. 2000). It is a quick and effective protocol for enriching membrane proteins without the need for ultracentrifugation. More complex membrane proteins (those with larger numbers of transmembrane domains) are better isolated using the membrane II kit, which enriches integral membrane proteins by treating a membrane preparation with sodium carbonate (Fujiki et al. 1982, Molloy et al. 2000); this protocol requires ultracentrifugation
- ReadyPrep protein extraction kit (cytoplasmic/nuclear) uses a proprietary buffer and differential centrifugation to isolate intact nuclei and a strongly chaotropic extraction buffer to quickly prepare highly enriched fractions of cytoplasmic and nuclear proteins from eukaryotic samples

ReadyPrep Protein Extraction Kit

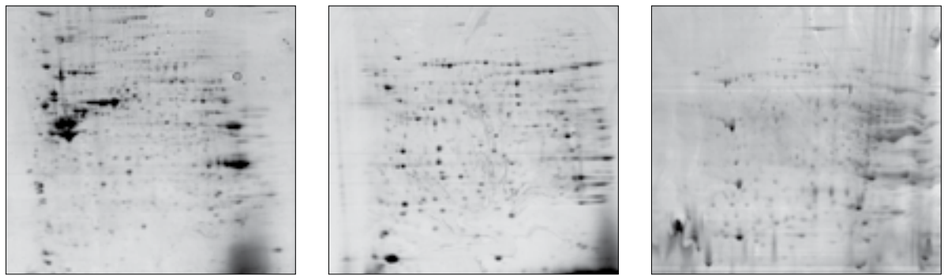


Fig. 2.4. Differences in 2-D patterns obtained using ReadyPrep protein extraction kits: signal (A), membrane I (B), and membrane II (C) kits. Mouse liver samples were extracted using each kit, and purified proteins were separated using 17 cm ReadyStrip pH 3–10 NL IPG strips and 8–16% gels. Overall spot patterns differ for A, B, and C even though all three kits isolate membrane proteins, indicating that each kit isolates different sets of proteins.

Products for Fractionation by Solubility/Hydrophobicity

- ReadyPrep sequential extraction kit is based on a published method (Molloy et al. 1998) that uses sequentially more highly solubilizing chaotrope and detergent mixtures. Applying each extracted fraction to a separate gel allows the resolution of more protein spots
- ReadyPrep protein extraction kit (soluble/insoluble) uses a different set of detergents to fractionate proteins on the basis of their solubility in detergents

The ReadyPrep sequential extraction kit and the ReadyPrep protein extraction kit (soluble/insoluble) can be used either independently or sequentially for even greater depth of coverage.

ReadyPrep Sequential Extraction Kit

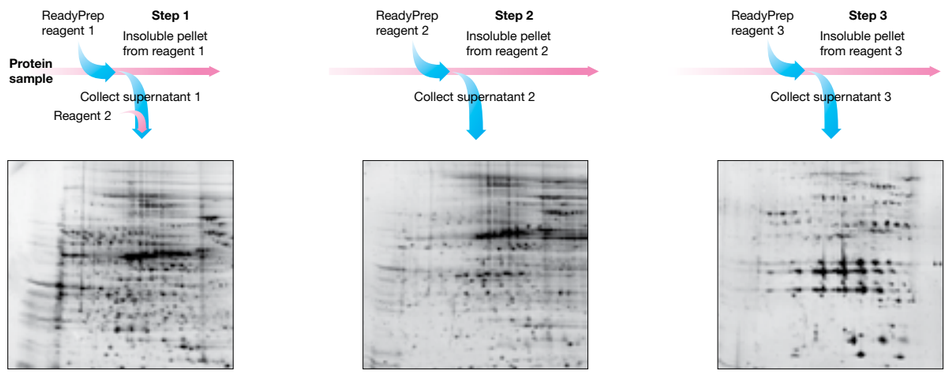


Fig. 2.5. Distribution of proteins based on differential solubility using the ReadyPrep sequential extraction kit. The generation of three fractions provides increased resolution of proteins on 2-D gels.

Fractionation by Solubility/Hydrophobicity

Proteins can be separated according to their solubility in different reagents using either chemical or chromatographic methods. Sequential extraction under different solvent conditions can be used to fractionate a protein sample based on solubility, and this strategy has also been used to prepare discrete fractions for analysis by 2-D electrophoresis (Lenstra and Bloemendal 1983, Weiss et al. 1992). Extraction using different detergents can also yield different protein fractions (Figure 2.5), and chromatographic methods that can be used include reverse-phase (Van den bergh and Arckens 2008) and hydrophobic interaction chromatography (McNulty and Annan 2009).

Fractionation by Protein Charge

Ion exchange chromatography has been used to reduce proteome complexity, enrich low-abundance proteins, and improve peptide mass fingerprints (Butt et al. 2001). This technique separates proteins according to their charge at various pHs. It is based on the reversible adsorption of proteins to a solid phase containing charged chemical groups. Cationic (+) or anionic (-) resins (Figure 2.6) attract molecules of opposite charge in the solvent. A variety of systems and media are available for ion exchange chromatography, but because elution involves gradient elution by washing the column with buffers of gradually increasing ionic strength or pH, a subsequent cleanup step must be included.

Products for Fractionation by Protein Charge

For prefractionation in a convenient kit format, Aurum™ AEX (anion exchange) and CEX (cation exchange) mini kits and columns employ ion exchange chromatography in an easy-to-use spin column format for fractionating and concentrating acidic and basic proteins from small sample volumes (<1 ml). Micro Bio-Spin 6 columns are included for salt removal from the fractionated samples. Requiring only 15–20 min operating time, Aurum ion exchange mini spin columns provide a quick, convenient, and reproducible sample preparation tool for 2-D electrophoresis, and their use can improve detection of low-abundance proteins (Liu and Paulus 2008).

Aurum Ion Exchange Kit

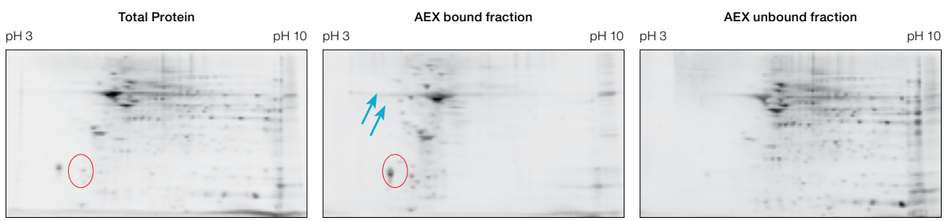


Fig. 2.6. Fractionation of rat brain tissue using Aurum ion exchange mini columns. Rat brain total protein extracts (3 ml) were loaded onto an Aurum AEX column and eluted. The unfractionated and fractionated samples were then treated with the ReadyPrep reduction alkylation and 2-D cleanup kits and separated by 2-D electrophoresis. Red circles indicate a group of protein spots with increased intensities after fractionation. Blue arrows show two representative spots detected only in the gels of the AEX bound fraction.

Fractionation by pI

Fractionation by pI, for example by liquid-phase IEF, may seem counterintuitive as a fractionation technique upstream of the first-dimension IEF separation. It can, however, improve downstream sample loading and separation on narrow- and micro-range IPG strips by eliminating proteins outside the pH region of interest (Figure 2.7). This unique separation method can also be coupled to analytical or preparative SDS-PAGE for a powerful, complementary first-dimension separation and enrichment strategy for high molecular weight, membrane, hydrophobic, or other proteins that are often underrepresented in IPG-based 2-D gels (Davidsson 2002, Hansson et al. 2004, Brobey and Soong 2007)[2].

[2] Liquid IEF introduces ampholytes that must be removed, for example with the ReadyPrep 2-D cleanup kit, before IEF in IPG strips.

Products for Fractionation by pI

The Rotofor®, Mini Rotofor, and MicroRotofor cells separate and concentrate proteins into discrete fractions by liquid-phase IEF. Following ampholyte removal and sample concentration with the ReadyPrep 2-D cleanup kit, each of the resulting liquid fractions can then be separated on narrow- or micro-range IPG strips.

Rotofor Family of Liquid-Phase IEF Cells

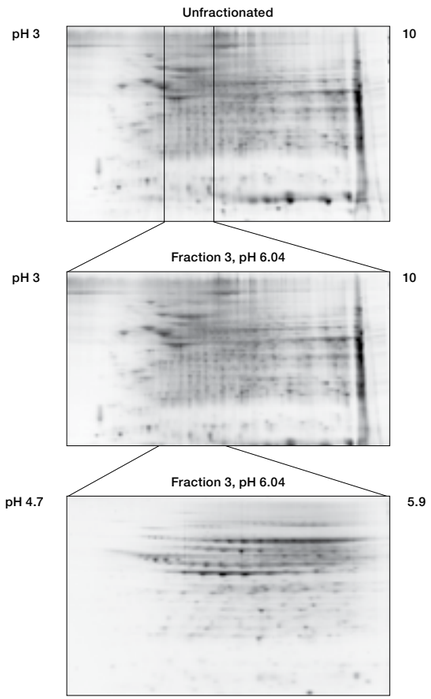


Fig. 2.7. Clean fractionation by pI. Mouse liver extract was fractionated using the MicroRotofor cell. 2-D separations of the unfractionated sample (120 µg) and fractions (30 µg) are shown. Prior to 2-D separation, samples were treated with the ReadyPrep 2-D cleanup kit to remove extra ampholytes. Note the clean pH boundaries of fraction 3 and the enrichment of proteins in the pH region it covers.

Fractionation by Size (MW)

Size-dependent separation is a powerful fractionation strategy in studies focused on a particular protein or protein family and their posttranslational modifications because these proteins tend to be of similar size (Fountoulakis and Juranville 2003). Proteins can be separated into size-dependent fractions by polyacrylamide gel electrophoresis (PAGE), particularly continuous-elution electrophoresis.

Products for Fractionation by Size (MW)

The Model 491 prep cell and mini prep cell perform size-dependent high-resolution fractionation of proteins by continuous-elution gel electrophoresis (using native PAGE or SDS-PAGE). The large sample capacity (50 µl–15 ml, and 0.5–500 mg protein) of these cells makes them particularly effective tools for the enrichment of low-abundance proteins (Zerefos et al. 2006, Xixi et al. 2006, Fountoulakis et al. 2004).

Model 491 Prep Cell and Mini Prep Cell

Depletion and Dynamic Range Reduction

One of the major difficulties facing proteomics is the issue of dynamic range, or the variation in abundance among sample proteins that typically spans several orders of magnitude. This range typically exceeds that over which proteins can be effectively detected and quantified. Various strategies have been developed for the reduction of sample dynamic range, and they have proven beneficial for the study of low-abundance proteins.

Depletion

Samples may be dominated by a few abundant proteins whose presence can obscure less abundant proteins and limit the capacity and resolution of the separation technique employed. This is particularly apparent for serum and plasma; the study of lower-abundance proteins from serum or plasma is often complicated by the presence of albumin and immunoglobulin G (IgG), which together contribute up to 90% of the total protein in a serum sample. These proteins obscure comigrating proteins and limit the amount of total serum protein that can be loaded on 2-D gels. To obtain meaningful results from serum samples, these proteins must be removed (Figure 2.8).

A strategy for specific depletion of abundant proteins by immunoaffinity chromatography has been widely used (Pieper et al. 2003, Roche et al. 2009, Tu et al. 2010, Ichibangase et al. 2008). Though this method is effective, the need for antibodies renders it expensive and limits its applicability to the specific sample type for which the antibodies were developed.

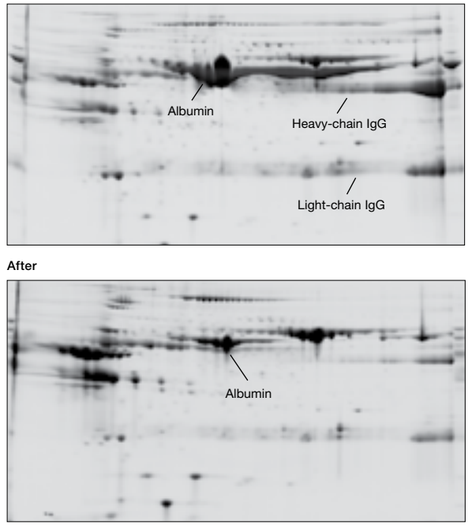


Fig. 2.8. Albumin and IgG removal from serum using the Aurum serum protein mini kit. Serum proteins were separated by 2-D electrophoresis before and after treatment with an Aurum serum protein mini column. Albumin and IgG are removed following treatment with the column, improving resolution of other protein species. Samples (100 µg) were focused on 11 cm ReadyStrip pH 5–8 IPG strips, then run on 8–16% gels.

Products for Depletion

Bio-Rad's Aurum Affi-Gel® Blue and Aurum serum protein mini kits represent a simple, low-cost alternative to immunodepletion. These kits use affinity chromatography to easily and effectively remove albumin (Affi-Gel Blue) or albumin and IgG (serum protein kit) in a single spin column.

Aurum Ion Exchange Kit

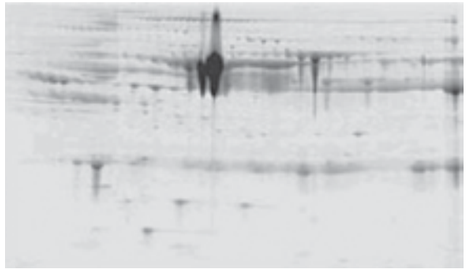
Dynamic Range Reduction

ProteoMiner™ protein enrichment technology uses a bead-based library of combinatorial peptide ligands that act as unique binders for proteins (Thalusiraman et al. 2005, Guerrier et al. 2006). When a complex sample is applied to the beads, abundant proteins saturate their specific ligands while the remaining proteins can be washed away. Low-abundance proteins are concentrated on their specific ligands and will be enriched relative to the abundant proteins following elution. In contrast to immunodepletion, ProteoMiner has no intrinsic specificity for any particular sample type and can be used to decrease high-abundance proteins in any sample that could benefit from such a treatment. The technology has been most widely applied to serum and plasma (Sennels et al. 2007); however, several examples of successful application of ProteoMiner to other samples have also been reported (Castagna et al. 2005, Guerrier et al. 2007, D'Ambrosio et al. 2008, Bandhakavi et al. 2009).

ProteoMiner protein enrichment kits:

- Decrease the amount of high-abundance proteins without immunodepletion, preventing the loss of proteins bound to high-abundance proteins
- Enrich medium- and low-abundance proteins that cannot be detected through traditional methods (Figure 2.9)
- Do not rely on a predefined set of antibodies, unlike immunodepletion products
- Are compatible with a variety of sample types
- Offer a convenient, easy-to-use format
- Can be used for differential expression analysis

Untreated



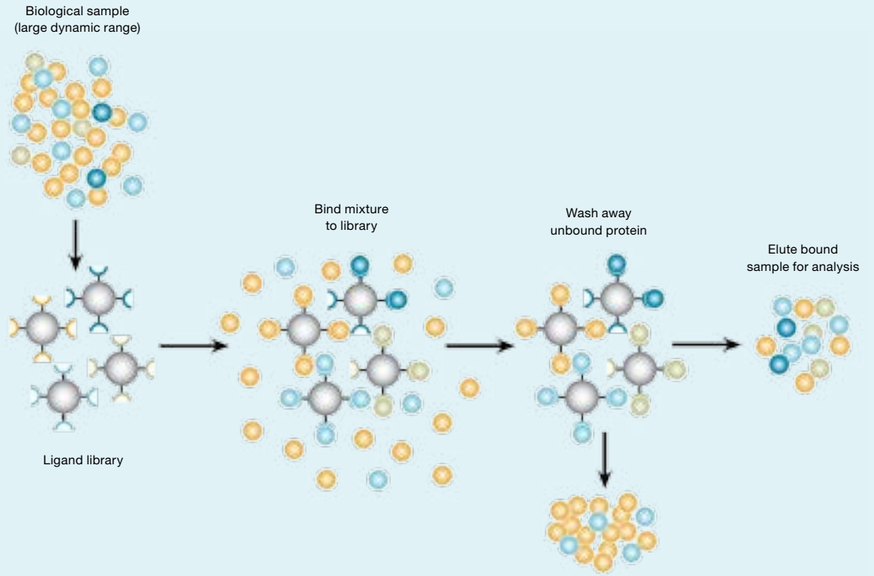
Treated

Fig. 2.9. The ProteoMiner protein enrichment kit improves resolution and spot counts in 2-D gels. In an untreated sample, albumin and other high-abundance proteins dominate the gel and obscure signals from less abundant proteins. On a gel generated using an equal amount of total protein from a treated serum sample, however, resolution is dramatically improved and a greater number of protein spots is visualized.

ProteoMiner Technology

ProteoMiner technology employs a combinatorial library of hexapeptides bound to a chromatographic support. Combinatorial synthesis creates a large library of unique hexapeptides, with each hexapeptide bound to a stationary support, or bead. Each bead, featuring a unique ligand, is expected to bind specifically to one or a small number of different proteins in a mixture, and the library of all possible sequences binds proteins up to the capacity of available beads.

When a complex biological sample is applied to the beads, high-abundance proteins saturate their ligands and excess proteins are washed away. In contrast, low-abundance proteins do not saturate their binding sites. Therefore, different samples retain relative expression levels similar to the original samples. Moreover, low-abundance proteins are enriched if the beads are eluted in a volume smaller than the original sample. The overall effect of ProteoMiner technology results in the bound and eluted material consisting of a significantly lower amount of total protein, thus allowing resolution of a greater diversity of species.



Depletion of plasma and serum samples by ProteoMiner technology. Each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. Samples are applied to the beads, allowing proteins to bind to their specific ligands. Proteins in excess are washed away, and those proteins bound to the beads are eventually eluted, allowing further downstream analysis.

Additional Resources

Samples can be prepared for 2-D electrophoresis using many other techniques. Consult Posch (2008) for more information on:

- Sample preparation basics (cell disruption, sample solubilization, protein assays, contaminant removal)
- Protein labeling techniques
- Fractionation using chemical reagents and chromatography
- Fractionation using electrophoresis methods
- Enrichment strategies for organelles, multiprotein complexes, and specific protein classes
- Application of sample preparation tools and fractionation strategies to study different biological systems

Sample Quantitation (Protein Assays)

Determine the concentration of protein in a sample (Berkelman 2008) by protein assay to:

- Ensure that the amount of protein to be separated is appropriate for the IPG strip length and visualization method
- Facilitate comparison among similar samples; image-based analysis is simplified when equivalent quantities of proteins have been separated

The most commonly used protein assays are visible assays, assays in which the presence of protein causes a visible color change that can be measured with a spectrophotometer (Sapan et al. 1999; Noble and Bailey 2009; see the Protein Assay Products and SmartSpec™ Plus Spectrophotometer sidebar). All protein assays utilize a dilution series of a known protein (usually bovine serum albumin or bovine γ-globulin) to create a standard curve from which the concentration of the sample is derived (for a protocol describing protein quantitation, refer to Part II of this guide).

The chemical components of the sample buffer and the amount of protein available for assay dictate the type of assay that may be used.

- **Bradford assays (Bradford 1976)** — based on an absorbance shift of Coomassie (Brilliant) Blue G-250 dye under acid conditions, when a redder form of the dye is converted into a bluer form upon binding to protein. The increase of absorbance at 595 nm is proportional to the amount of bound dye and, therefore, to the amount (concentration) of protein present in the sample. In comparison to other protein assays, the Bradford protein assay is less susceptible to interference by various chemicals that may be present in protein samples, with the exception of elevated concentrations of detergents like SDS. The response of the Bradford protein assay is only slightly affected by urea, thiourea, and CHAPS in concentrations up to 1.75 M, 0.5 M, and 1% (w/v), respectively
- **Lowry (Lowry et al. 1951)** — combines the reactions of cupric ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. The Lowry method is based on the reaction of Cu^{+}, produced by the peptide-mediated reduction of Cu^{2+}, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction). The Lowry assay is intolerant of thiourea, reductants such as DTT, and chelating agents such as EDTA
- **BCA (bicinchoninic acid, Smith et al. 1985)** — BCA reacts directly with Cu^{+} (generated by peptide-mediated reduction of Cu^{2+}) to produce a purple end product. The reagent is fairly stable under alkaline conditions and can be included in the copper solution to allow a one-step procedure. Like the Lowry assay, the BCA assay is intolerant of thiourea, reductants such as DTT, and chelating agents such as EDTA

2-D sample solutions typically contain reagents that interfere with all of the assays described above. The Bradford assay may be used on samples that are concentrated enough to be diluted with water so that urea, thiourea, and CHAPS are no longer present at interfering levels (typically at least fourfold). Otherwise, modified assay procedures may need to be employed (see the Protein Assay Products sidebar).

Protein Assay Products

Protein concentration in 2-D sample solutions is best measured using the *RC DC*™ protein assay, a modification of the Lowry assay that incorporates a precipitation step that removes reducing agents and detergents. For more information on protein quantitation using visible assays, refer to Bio-Rad bulletin 1069.

Table 2.3. Bio-Rad protein assay selection guide.

	Quick Start™ Bradford	Bio-Rad	*DC*™	*RC DC*
Method				
Bradford	•	•	–	–
Lowry	–	–	•	•
Description	One-step determination; not for use with SDS-containing samples	Standard Bradford assay, not to be used with elevated levels of detergents (>0.1% SDS)	Detergent compatible (*DC*); Lowry assay modified to save time and to be more accurate	Reducing agent and detergent compatible (*RC DC*)
Standard-concentration Assay				
Sample volume	100 µl	100 µl	100 µl	100 µl
Linear range	0.125–1.5 mg/ml	0.125–1.5 mg/ml	0.125–1.5 mg/ml	0.2–1.5 mg/ml
Low-concentration Assay				
Sample volume	1 ml	800 µl	200 µl	200 µl
Linear range	1.25–25 µg/ml	1.25–25 µg/ml	5–250 µg/ml	5–250 µg/ml
Microplate assay volume	5 µl	10 µl	5 µl	**
Minimum incubation	5 min	5 min	15 min	15 min
Assay wavelength	595 nm	595 nm	650–750 nm	650–750 nm

SmartSpec Plus Spectrophotometer

The color change observed in protein assays is measured using a spectrophotometer. Bio-Rad's SmartSpec Plus spectrophotometer has preprogrammed methods for protein quantitation and a working wavelength range of 200–800 nm. It can be used for routine applications such as:

- Quantitation of proteins via the Bradford, Lowry, and BCA assay methods
- Quantitation of DNA, RNA, and oligonucleotides
- Monitoring bacterial culture growth
- Simple kinetic assays
- Wavelength scans with peak detection

Features built into the SmartSpec assay methods facilitate data collection and present a complete analysis of assay results. Bio-Rad also offers compatible quartz and UV-transparent plastic cuvettes.

SmartSpec Plus Spectrophotometer

CHAPTER 3

The First Dimension: Isoelectric Focusing (IEF)

Protein Separation by Isoelectric point (pI)

The first-dimension separation of 2-D electrophoresis is IEF, where proteins are separated on the basis of differences in their pI. The pI of a protein is the pH at which it carries no net charge, and it is a characteristic that is determined by the number and types of charged groups the protein carries.

Proteins are amphoteric molecules, which carry a positive, negative, or zero net charge depending on the pH of their environment. For every protein, there is a specific pH at which its net charge is zero (its pI). Proteins show considerable variation in pI, though pI values usually fall in the range of pH 3–12, with the majority falling between pH 4 and pH 8. A protein is positively charged at pH values below its pI and negatively charged at pH values above its pI (Figure 3.1).

For IEF, a protein is placed in a medium with a pH gradient and subjected to an electric field. In response to the field, the protein moves toward the electrode with the opposite charge. Along the way, it either picks up or loses protons. Its net charge and mobility decrease until the protein eventually arrives at the point in the pH gradient equal to its pI. There, the protein is uncharged and stops migrating (Figure 3.2). If, by diffusion, it drifts away from the point in the gradient corresponding to its pI, it acquires charge and is pulled back. In this way, proteins condense, or are focused, into sharp bands in the pH gradient at their characteristic pI values.

IEF proceeds until a steady state is reached. Proteins approach their pI values at different rates but remain relatively fixed at those pH values for extended periods. This is in contrast to conventional electrophoresis (for example, polyacrylamide gel electrophoresis, or PAGE), where proteins continue to move through the medium until the electric field is removed. Moreover, in IEF, proteins migrate to their steady-state positions from anywhere in the system. IEF for 2-D electrophoresis is performed under denaturing conditions so that proteins are completely disaggregated and all charged groups are exposed to the bulk solution. Consequently, resolution is best under denaturing conditions. Complete denaturation and solubilization are required to minimize aggregation and intermolecular interactions, thus ensuring that each protein is present in only one configuration.

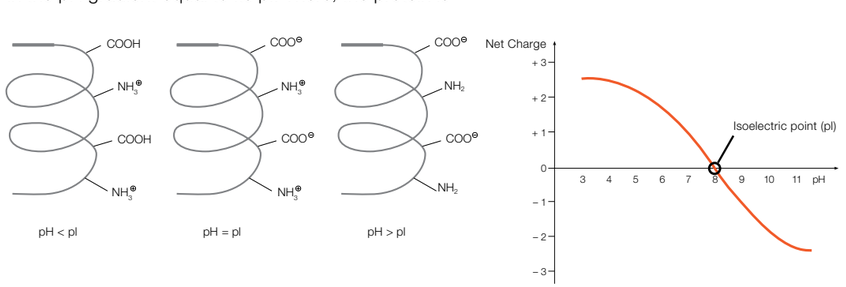


Fig. 3.1. Dependence of protein net charge on the pH of its environment. The pH at which the net charge is 0 is the isoelectric point (pI).

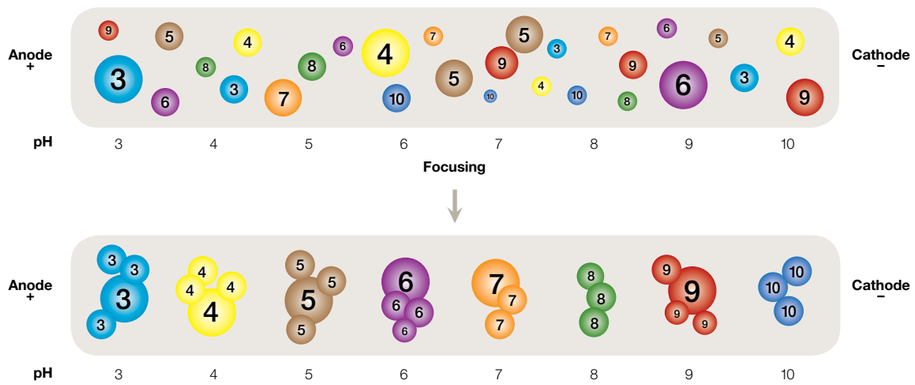


Fig. 3.2. Principle of IEF. A mixture of proteins is separated in a pH gradient and within an electric field according to each protein's pI and independently of its size. The proteins migrate until they reach their pI.

IEF Media: IPG Strips vs. Carrier Ampholytes

IEF for 2-D electrophoresis is most commonly performed using immobilized pH gradient (IPG) strips. As their name implies, IPG strips contain buffering groups covalently bound to a polyacrylamide gel strip to generate an immobilized pH gradient. The pH gradients are created with sets of acrylamido buffers, which are derivatives of acrylamide containing both reactive double bonds and buffering groups. The general structure is CH_2=CH–CO–NH–R, where R contains either a carboxyl [–COOH] or a tertiary amino group (for example, –N(CH_3)$_2$). These acrylamide derivatives are covalently incorporated into polyacrylamide gels at the time of casting and can form almost any pH gradient (Righetti 1990).

IPG strips are:

- Supplied commercially and ready to use
- Prepared on a plastic backing to simplify handling
- Highly reproducible and stable over even extended IEF runs (Bjellqvist et al. 1982)
- Available in a wide variety of pH gradients and lengths (see the ReadyStrip™ IPG Strips sidebar)

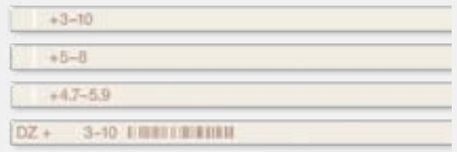
Historically, first-dimension IEF was performed using carrier ampholyte–generated pH gradients and tube gels. This type of first dimension has been largely superseded by the use of IPG strips for the following reasons:

- Carrier ampholyte tube gels must be cast by the user
- Carrier ampholyte–generated pH gradients drift over time and are, therefore, not as reproducible as immobilized pH gradients
- Carrier ampholytes are complex chemical mixtures, and batch-to-batch variations affect the characteristics of the pH gradient
- Narrow pH gradients and gradients encompassing the extremes of the pH range (below pH 4 and above pH 9) cannot be accommodated
- Tube gels can be difficult to handle

ReadyStrip IPG Strips

IPG strips simplify first-dimension separations by immobilizing the pH gradient on an easy-to-handle support strip. ReadyStrip IPG strips are available in a wide selection of pH gradients and strip lengths (from 7 to 24 cm) to fit Bio-Rad vertical electrophoresis cells and gels. Premade ReadyStrip IEF buffers are also available for convenience and maximum reproducibility.

Relative separation. Relative focusing power expresses the enhanced resolution expected in the first dimension when using IPG strips of different lengths or pH ranges. The 7 cm pH 3–10 IPG strip is arbitrarily assigned a baseline focusing power of 1.0 to calculate the relative focusing powers of the other strips.



ReadyStrip IPG strips are preprinted to indicate anode (+) and pH range; in addition, a bar code is printed on the 24 cm strip.

ReadyStrip IPG strip pH ranges.

	pH							Relative Focusing Power					ReadyStrip IEF Buffer					
Strip Range*	3	4	5	6	7	8	9	10	7 cm	11 cm	17 cm	18 cm	24 cm	3–10	7–10	3.9–5.1	4.7–5.9	5.5–6.7	6.3–8.3
Broad Range																			
3–10									1×	1.6×	2.4×	2.6×	3.4×	•					
3–10 nonlinear (NL)									1×	1.6×	2.4×	2.6×	3.4×	•					
Narrow range																			
3–6									2.3×	3.7×	5.7×	6.0×	8.0×	•					
5–8									2.3×	3.7×	5.7×	6.0×	8.0×	•					
7–10									2.3×	3.7×	5.7×	6.0×	8.0×		•				
4–7									2.3×	3.7×	5.7×	6.0×	8.0×	•					
Micro range																			
3.9–5.1									5.8×	9.2×	14.2×	15.0×	20.0×			•			
4.7–5.9									5.8×	9.2×	14.2×	15.0×	20.0×				•		
5.5–6.7									5.8×	9.2×	14.2×	15.0×	20.0×					•	
6.3–8.3									3.5×	5.5×	8.5×	9.0×	12.0×						•

* Strips are designed with sufficient overlap to allow spot matching while limiting the extent of redundant data.

Selection of IPG Strips

When selecting the IPG strip, consider both the pH gradient and strip length, as both determine the resolution in the final 2-D gel (see the ReadyStrip IPG strips sidebar).

Choice of pH Gradient

IPG strips are available in various pH gradients (see the table in the ReadyStrip IPG Strips sidebar). The pH gradients are linear (pH varies in a linear manner with respect to length of the strip) except in the case of nonlinear pH 3–10 gradients (NL, see the Estimation of pI sidebar).

- Use broad-range strips (for example, pH 3–10) for an overview of the spot distribution along the pH gradient and for comparing different sample preparation strategies. Since many proteins focus in the middle of the pH range 3–10, using NL gradients can improve resolution of proteins in the middle of that range and compress the width of the extreme pH ranges at the ends of the gradients
- Use narrow- and micro-range gradients for greater resolution (there is a larger separation distance, more cm of gel, per pH unit). With the exclusion of proteins outside the pH range of the strip, more total protein mass can be loaded per strip to also allow detection of more proteins
- Use overlapping pH ranges to increase resolution by expanding a small pH range across the entire width of a gel (Figure 3.3). This also allows the creation of composite gels by matching spots from the overlapping regions using imaging software

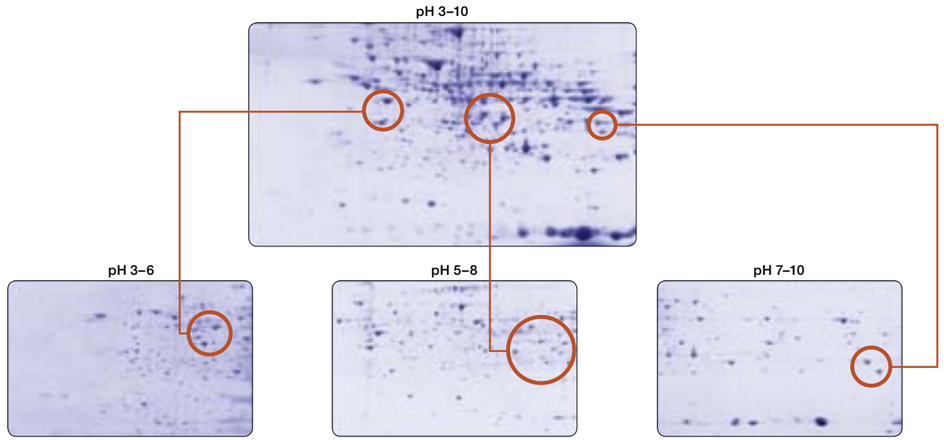


Fig. 3.3. A mouse liver sample was extracted in a urea-thiourea-CHAPS solution. The extract was run in a single PROTEAN® i12™ IEF cell run on twelve 11 cm ReadyStrip IPG strips simultaneously at each of the following pH ranges: 3–10, 3–6, 5–8, and 7–10. Each pH gradient was run in triplicate. The second dimension for each IPG strip was run in 8–16% gradient Criterion™ precast gels that were stained with Bio-Safe™ Coomassie stain. The above figure shows a representative gel image for each pH range.

Choice of IPG Strip Length

IPG strips are available in a variety of lengths that match the size of most commercial second-dimension vertical electrophoresis systems. Shorter strips match mini-format systems, and longer strips match large-format systems. Deciding which strips to use depends on the requirements for speed, sample volume, resolution, and throughput (see Chapter 4 for more details on selecting size format for 2-D electrophoresis):

- Use shorter strips and mini-format gels for fast, convenient sample screening or method development
- Use longer strips for the best separation with higher protein loads and for maximum resolution. The longest IPG strips and large-format gels have a large area to resolve protein spots; however, they take a long time to run
- Combine different size formats for various benefits. For example, use a mini-format system for rapid optimization of sample preparation methods, then switch to a large format for thorough assessment of a complex sample and identification of proteins of interest. In many cases, a mini system and narrow-range IPG strips can then be used to focus in on proteins of interest
- Use overlapping narrow- and micro-range IPG strips to increase the effective length of pI resolution. When three narrow-range overlapping ReadyStrip IPG strips (pH 3–6, 5–8, 7–10) are used with the Criterion system, for example, the resolution in the first dimension (11 cm strip, pH 3–10 NL) is increased from 11 to 26 cm. When four micro-range strips are used, the resolution in the first dimension is expanded from 11 to 44 cm

Estimation of pI

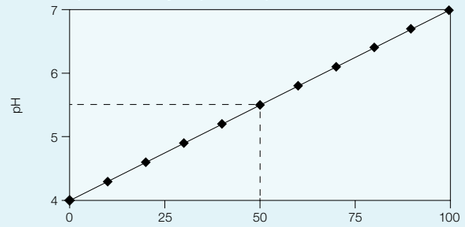
The pI of a protein is a useful parameter for protein characterization. With purified proteins, pI can be determined by IEF using IPG strips, usually under denaturing conditions. Using linear IPG strips, the pH gradient can be assumed to extend linearly between the pH extremes. Knowing the length and pH range of the IPG strip implies that experimental pI values can be assigned with a high level of accuracy (see figure). Protein pI estimations can also be made using NL IPG strips, assuming the pH profile of the IPG strip is available from the manufacturer; without the exact pH profile of the strip, the pI estimate will be less accurate.

For pI estimation, stain the IPG strips after IEF, for example with Bio-Safe Coomassie blue stain, and then plot the migration distance along the length of the IPG strips of each of the protein standards. Graph A shows the pH gradient along the length of a linear pH 4–7 IPG strip. To determine the pI of an unknown, simply determine the band position (as a percentage of gel length) and read the pI from the graph. In the example, a band positioned at 50% of the gel length will have an estimated pI of 5.5.

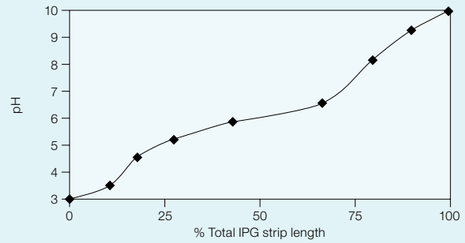
The same strategy can be applied for protein spots on 2-D gels, but with less accuracy due to swelling or shrinkage of the 2-D gel. It may also be difficult to define the start and end positions of the IPG strip on stained 2-D gels.

With knowledge of experimental pI and molecular weight values (see Chapter 4 for details about molecular weight estimation), it is possible to make comparisons with the calculated values derived after protein spot identification using mass spectrometry. The calculation of theoretical pI values is possible with software tools available on the Internet, for example at **http://web.expasy.org/compute_pi**. If the values differ significantly from each other, this may indicate a false identification or the identification of a fragment of the respective protein. However, differences in pI or molecular weight can also suggest posttranslational modifications, such as phosphorylation or glycosylation. The detection of posttranslational modifications is a unique strength of gel-based proteomics. These modifications offer information about the function, regulation, and cellular location of proteins.

A. Linear pH 4–7 ReadyStrip IPG strip



B. Nonlinear pH 3–10 ReadyStrip IPG strip



Estimating the pI of a protein from its position along an IPG strip. A, By plotting the pH of an IPG strip as a function of its length, the pI of a protein may be derived from its focused position on that strip. In the example shown, the pI of a protein that migrates across 50% of the strip length is 5.5. **B**, pH profile of Bio-Rad ReadyStrip nonlinear pH 3–10 IPG strips.

Sample Application

Commercial IPG strips are dehydrated and must be rehydrated to their original gel thickness (0.5 mm) before use. The protein sample can be applied to the IPG strip either during or after rehydration, and rehydration can be done in either disposable rehydration/equilibration trays or directly in the focusing tray. Sample application during rehydration is the easiest and, in most cases, most efficient way to apply sample. In some instances, however, it is best to rehydrate the IPG strips and then apply sample through sample cups while current is applied (cup loading) (Table 3.1). Each method is discussed in the following sections.

The rehydration solution generally contains the following components to maintain protein solubility and allow tracking of the separation (see Part II, Methods for recipes):

- **Chaotrope** — urea (8 M or up to 9.8 M if necessary for sample solubility), with or without 2 M thiourea
- **Detergent** — nonionic or zwitterionic detergent such as CHAPS, Triton X-100, or NP-40 at 0.5–4% (w/v)
- **Reducing agent** — 20–100 mM DTT
- **Ampholytes** — 0.2% (w/v), usually pH 3–10; concentrations up to 1% (w/v) may be used, though this reduces the voltage and results in correspondingly longer runs
- **Tracking dye** — a trace of bromophenol blue to render the IPG strip more visible for simplified handling and act as a tracking dye for confirmation of focusing

The composition of the rehydration solution should also resemble the composition of the sample solution in terms of the additives present to aid solubility. If the sample was prepared using thiourea, the rehydration solution should also contain thiourea. Likewise, the same detergent should be used. Otherwise, transition from one solution to the other can cause precipitation of proteins that are soluble in the sample solution but not in the rehydration solution

Sample Application during Rehydration

In this option, the sample is prepared in, or diluted into, rehydration solution and introduced to the IPG strip at the time of rehydration. As the strips hydrate for at least 12 hr, proteins in the sample are absorbed and distributed over the entire length of the strip (Rabilloud et al. 1994, Sanchez et al. 1997).

Sample application can be in either the absence (passive) or presence (active) of applied voltage:

- Active rehydration is performed in the IEF cell. A low voltage (30–100 V) is applied, and proteins enter the gel matrix under current as well as by absorption. Active rehydration with the sample is believed to promote the entry of large proteins into the strip by applying an electrical “pull”
- In passive rehydration, proteins enter the gel by absorption only. This allows efficient use of equipment, since strips can be rehydrated in sample rehydration trays while other samples are focused in the IEF cell

Table 3.1. Advantages and disadvantages of sample loading methods.

Method	Advantages	Disadvantages
In-gel rehydration	Simple sample application	Poor resolution of basic proteins
	No precipitation at the point of sample application	
	Accommodates dilute samples and larger protein loads	
Passive	Focusing can follow rehydration without manual intervention if performed within the IEF instrument	Not all proteins, particularly large or hydrophobic proteins, will be taken up
Active	More effective with certain proteins, particularly those of high molecular weight	Rehydration must occur within the IEF instrument
Cup loading	More effective for basic proteins	Setup more complicated; the cup must form a seal with the IPG strip
	Can improve resolution at extremes of the pH gradient (the end opposite the point of application)	High protein loads are difficult to accommodate; concentrated samples are required.
		Sample precipitation may occur at the point of application

Whether IPG strips are rehydrated actively or passively, they must be incubated with sample for at least 12 hr prior to IEF. This gives high molecular weight proteins time to enter the gel after the gel has become fully hydrated and the pores have attained full size.

Sample application during rehydration works because IEF is a steady-state technique; therefore, proteins migrate to their pI independently of their initial positions. The advantages of these approaches over cup loading are:

- Simplicity
- Reduced risk of sample precipitation, which can occur with cup loading at the sample application point if sample concentration is too high (Rabilloud 1999)
- Shorter focusing times can be used because the sample proteins are in the IPG strip prior to IEF
- Large amounts of protein can be loaded

Sample Application by Cup Loading

To apply samples after IPG strip rehydration, the sample is loaded into sample cups positioned on the rehydrated strip. This technique can be more challenging than in-gel sample loading from a technical standpoint, but it can be beneficial in the following cases (Cordwell et al. 1997, Görg et al. 2000):

- When samples contain high levels of DNA, RNA, or other large molecules, such as cellulose
- When running acidic and basic IPG strips; for example, pH 7–10
- When running micro-range IPG strips spanning ~1 pH unit
- For samples that contain large amounts of glycoproteins

Setup for IEF

For IEF, the rehydrated IPG strips are placed into the focusing tray. The orientation (gel-side up or gel-side down) of the IPG strip in the focusing tray is largely determined by the sample loading method employed:

- Cup loading requires gel-side up strip placement so that the sample cup may be placed in contact with the gel surface
- In-gel sample loading is conducted gel-side down. If the IEF cell is programmed for an unattended start following rehydration, IEF must be conducted gel-side down as well
- If in-gel sample loading is performed in the rehydration/equilibration tray, IEF may be performed either gel-side up or gel-side down. This is largely a matter of user preference, though improved resolution may be observed with the gel-side up configuration, particularly with higher protein loads

In addition, electrode wicks may be placed between the electrode in the focusing tray and the IPG strip in either running configuration. Electrode wicks serve as a sink for ionic sample contaminants and proteins with pIs outside the pH range of the IPG strip used. They also prevent drying of the ends of the IPG strips during IEF. In some cases, however, the use of electrode wicks has little effect on separation quality, and they may be omitted for convenience in either running configuration if satisfactory results are obtained in their absence.

Power Conditions for IEF

The pH gradient and the electric field strength both influence the time required to reach steady state and the resolution of the separation. The electric field strength is determined by the length of the IPG strip and the applied electrical field. In general, narrow pH ranges yield higher resolution and require higher voltages and more time to reach a steady state.

Longer IPG strips can withstand higher programmed voltages and require an increased number of volt-hours (Vh) for proper focusing. Vh are the product of voltage and time. Because the actual voltage reached is current dependent and the maximum programmed voltage may not be reached, programming the IEF cell with Vh can better ensure that samples receive a consistent number of volts.

The electrical conductivity of the system changes during an IEF run. At the beginning, the current is relatively high because of the large number of charge carriers present. As the proteins and ampholytes move toward their pIs, and as ionic contaminants move to the ends of the IPG strip, the current gradually decreases. When the proteins reach their final positions in the pH gradient, there is little ionic movement in the system; the voltage reaches a maximum, and the current reaches a minimum.

Focusing should occur with a gradual increase in voltage followed by a prolonged focusing phase at the maximum voltage advisable for the IPG strip length used and until a set number of Vh have accumulated. The optimum duration depends on the length of the IPG strip and the pH gradient. Current is generally limited to 50 µA per IPG strip, and a streamlined one-step protocol is adequate in most circumstances, as the voltage will rise gradually without the need for a phased focusing protocol with programmed voltage ramping. A more gradual focusing protocol may be advisable in circumstances of heavy protein load, for some narrow- and micro-range IPG strips, or when there are high levels of charged contaminants present. Since the duration of the prolonged focusing phase is specified in Vh, the actual duration may vary depending on the average voltage during focusing. Focusing may conclude at different times for IPG strips run at the same time with the same protocol. It is important, therefore, to include a hold step during which the IPG strip is held at a relatively low voltage to maintain focusing until the IPG strip can be removed from the instrument.

Electrical current generates heat, which limits the magnitude of the electric field that can be applied and the ionic strength of the solutions that can be used. Thin gels dissipate heat better than thick gels and thus better withstand the high voltage that offers higher resolution. Also, as mentioned above, the current drops to a constant low value as focusing reaches a steady state.

PROTEAN i12 IEF System

The PROTEAN i12 IEF system allows multistep runs at durations set by either time or Vh. Recommended starting electrical conditions and voltage ramping options are provided in Part II of this guide; however, sometimes the number of Vh required to complete a run must be determined empirically in a time course experiment. The optimum Vh depends on the sample and the composition of the sample solution as well as the pH gradient of the IPG strip. A more complex sample or different sample buffer might change the Vh required. The time needed to achieve the programmed Vh depends on the pH range of the IPG strip as well as sample and buffer characteristics.

Other IEF cells only support running identical pH gradients and similar samples in batches because the programmed current and voltage are spread across all lanes. If different pH ranges or samples with varying conductivity are run at the same time, the electrical conditions experienced by individual IPG strips are different. This may expose some strips to more or less current than desired, since the total current limit is averaged over the tray. The individual lane control provided by the PROTEAN i12 IEF cell, however, ensures that the current limit is not exceeded in any IPG strip, even in situations where conductivity differs significantly among samples run at the same time.

The PROTEAN i12 IEF cell also allows each lane to be programmed individually, making it possible to run different protocols in different lanes. The flexibility of this system allows running different experiments at once or varying conditions within an experiment to allow optimization in fewer runs. The system also results in better reproducibility because focusing conditions are not influenced by other samples in the run.

PROTEAN i12 IEF Cell and Accessories

CHAPTER 4

The Second Dimension: SDS-PAGE

Protein Separation by Size

The second-dimension separation is by protein size (mass) using SDS-PAGE. The proteins separated in IPG strips by IEF in the first dimension are applied to polyacrylamide gels and separated a second time by SDS-PAGE (Figure 4.1).

A two-step equilibration process prepares the proteins for SDS-PAGE. The proteins are complexed with SDS, reduced with DTT, and then alkylated with iodoacetamide. Treatment of the proteins with SDS yields protein-SDS complexes with a consistent charge-to-mass ratio. When electrophoresed through a polyacrylamide gel, these complexes migrate with a mobility that is related logarithmically to mass. As the proteins migrate through the gel, the pores of the gel sieve proteins according to size.

Selection of Polyacrylamide Gels

Polyacrylamide gels are prepared by free radical polymerization of acylamide and a comonomer crosslinker such as bis-acrylamide. By convention, gels are characterized by two parameters that determine pore size: total monomer concentration (%T, in g/100 ml) and weight percentage of crosslinker (%C). SDS-PAGE gels typically have a %C of 2.7%, and the %T is varied to give separation characteristics appropriate to the experimental needs. %T determines the relative pore size of the resulting polyacrylamide gel, with higher %T resulting in smaller pores and separation characteristics more appropriate for smaller proteins.

Gels are either purchased as commercial precast gels or cast in the laboratory using unpolymerized monomer and buffer components. Precast gels are available in smaller formats to fit commercially available electrophoresis cells. These are appropriate for the second dimension when the first dimension is run on 7 cm or 11 cm IPG strips. Larger second dimensions are generally run on lab-cast gels.

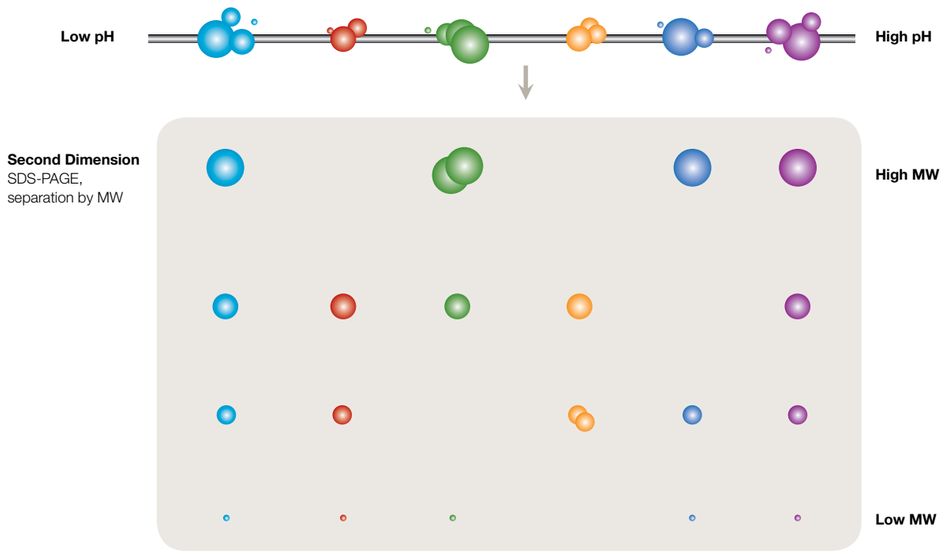


Fig. 4.1. Separation of proteins by SDS-PAGE after separation by IEF. The IPG strip containing proteins already separated by pI is applied to the top of a polyacrylamide gel. The proteins are then separated according to size (MW) by SDS-PAGE.

Choice of Gel Percentage (Composition)

Gels for SDS-PAGE are made with either a single, continuous %T throughout the gel (single-percentage gels) or a gradient of %T (gradient gels). Gradient gels are cast with acrylamide concentrations that increase from top to bottom so that the pore size decreases as proteins migrate into the gels. Single percentage gels are cast in the laboratory by simply pouring the appropriate percentage of acrylamide, along with bis-acrylamide, buffer, initiator, and catalyst, into a gel cassette prepared using glass plates and spacers clamped together. The mixture is poured into the cassette and allowed to polymerize. A stacking layer is not necessary for second-dimension gels. Gradient gels may also be cast in the laboratory using solutions of differing acrylamide percentage and a gradient maker. Typical gel compositions are 7.5–20% for single-percentage gels, and 4–15% to 10–20% for gradient gels.

Use protein migration charts and tables to select the gel type that offers optimum separation of your sample (Figure 4.2):

- Use single-percentage gels to separate bands of similar size. Since optimum separation occurs in the lower half of the gel, choose a percentage in which the protein of interest migrates to the lower half of the gel
- Use gradient gels to separate a broad range of protein sizes. Gradient gels allow resolution of both high- and low-molecular weight bands on the same gel. The larger pore size towards the top of the gel permits resolution of larger molecules, and decreasing pore sizes toward the bottom of the gel restrict excessive separation of small molecules. Gradient gels are often the most appropriate choice for 2-D electrophoresis, which is most often applied to complex samples with proteins spanning a large size range
- For new or unknown samples, use a broad gradient gel (for example, 4–20 %T or 8–16 %T or Bio-Rad's Any kD™ [3] formulation) for a global evaluation of the sample, and then move to an appropriate single-percentage gel for more detailed investigation of a particular size range of interest

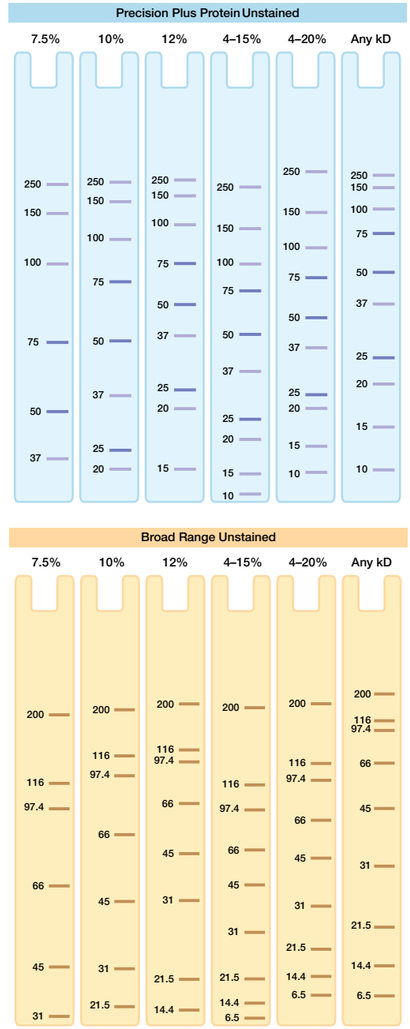


Fig. 4.2. Examples of migration charts. The protein standards were run on Mini-PROTEAN® TGX™ gels.

[3] Bio-Rad's Any kD formulation provides separation of 10–250 kD proteins, with the best resolution in to the 20–100 kD range. These gels are useful for screening samples or for 2-D applications aimed at rapid protein analysis.

Vertical Electrophoresis Systems for SDS-PAGE

The electrophoresis systems offered by Bio-Rad for second-dimension electrophoresis are detailed in the table.

- The Mini-PROTEAN system includes the Mini-PROTEAN Tetra cell (with a capacity of up to four gels) and the high-throughput Mini-PROTEAN® 3 Dodeca™ cell (for running up to 12 gels). These systems accommodate 7 cm IPG strips and are compatible with handcast or precast Mini-PROTEAN gels (8.6 × 6.7 cm)
- The Criterion™ system includes the Criterion cell (for 1–2 gels) and the high-throughput Criterion Dodeca cell (for 1–12 gels); both cells accommodate Criterion precast gels (13.3 × 8.7 cm) and 11 cm IPG strips
- The PROTEAN II and PROTEAN Plus Dodeca systems accommodate 17, 18, or 24 cm IPG strips. The PROTEAN II system provides the ability to choose the glass plates, spacer, and sandwich clamps to cast two gel lengths: 16 or 20 cm. The PROTEAN Plus Dodeca cell allows maximum throughput for 2-D electrophoresis, with the capability to run up to 12 2-D gels at a time

Bio-Rad's vertical electrophoresis systems.

	Mini-PROTEAN System	Criterion System	PROTEAN II System	PROTEAN Plus System
Cells	Mini-PROTEAN Tetra Cell Mini-PROTEAN Dodeca Cell	Criterion Cell Criterion Dodeca Cell	PROTEAN II XL Cell PROTEAN II XL Multi Cell	PROTEAN Plus Dodeca Cell
Number of gels	1–4 1–12	1–2 1–12	1–4* 1–6	1–12
Gel formats	Mini-PROTEAN precast Mini-PROTEAN handcast	Criterion precast Criterion empty cassettes	PROTEAN II handcast	PROTEAN Plus handcast
IPG strip length	7 cm	11 cm	17 cm	18 and 24 cm

* For 2-D applications running a maximum of 2 gels at a time is recommended.

PowerPac™ Power Supplies

Power supplies are required to meet the power requirements of numerous applications. The choice of power supply for second-dimension PAGE usually depends on the size and number of gels being run:

- Use the PowerPac Basic or PowerPac HC high-current power supply for mini-format vertical PAGE applications
- Use the PowerPac HV high-voltage or PowerPac Universal power supply for large-format vertical PAGE applications
- Use the PowerPac HC power supply for applications that require high currents, such as PAGE with the high-throughput Dodeca cells

PowerPac HC High-Current Power Supply

PowerPac Basic Power Supply

PowerPac Universal Power Supply

PowerPac HV High-Voltage Power Supply

Choice of Gel Size

The choice of gel size depends on the same factors determining the length of IPG strip used, namely speed, resolution, and throughput (see the Choice of IPG Strip Length section):

- Mini-format systems accommodate short IPG strips (7 cm) and mini-format gels. The short separation distance of the gels maximizes the electrical field strength (V/cm) to yield rapid separations with moderate resolution. Use mini gels and systems for rapid analysis and method development
- Midi gels and midi-format systems accommodate 11 cm IPG strips and are slightly larger (both in width and length) gels. They still offer rapid runs, but because of the longer separation distance, they provide better coverage than mini-format gels
- Large-format systems accommodate 17–24 cm IPG strips and large gels and offer the maximum resolution possible; however, large gel sizes require longer run times

Choice of Buffer System

The pH and ionic composition of the buffer systems used to prepare the gels and samples and to fill the electrode reservoirs determine the power requirements and heavily influence the separation characteristics of a polyacrylamide gel. Different buffer systems also vary widely in their stability.

The most common buffer system for second-dimension SDS-PAGE is the Tris-HCl system described by Laemmli (Laemmli 1970). The reagents are inexpensive and readily available, and the precast gels are also readily available in a wide variety of gel percentages. The system is robust and highly tolerant of high sample loads. However, Tris-HCl resolving gels are prepared at pH 8.6–8.8; at this basic pH, polyacrylamide slowly hydrolyzes to polyacrylic acid, which can compromise separation. For this reason, Tris-HCl gels have a relatively short shelf life. In addition, the gel pH can rise to pH 9.5 during a run, causing proteins to undergo deamination and alkylation, thereby diminishing resolution and complicating post-electrophoresis analysis.

To alleviate these shortcomings, a number of alternative buffer systems have been developed. For example, bis-Tris, Tris-acetate, and other proprietary buffer systems (see the Precast Gels for Second-Dimension SDS-PAGE sidebar) offer extended shelf life as well as other separation characteristics unique to their formulations.

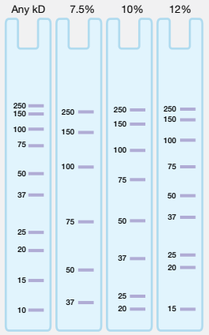
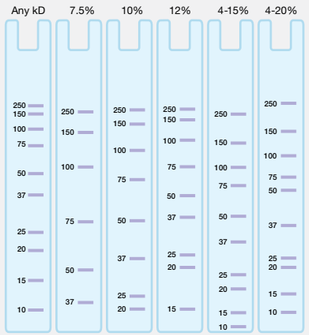
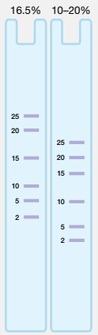
High-quality precast gels are preferred for high-throughput applications. They provide savings in time and labor, and the precision-poured gradients result in reproducibility among runs.

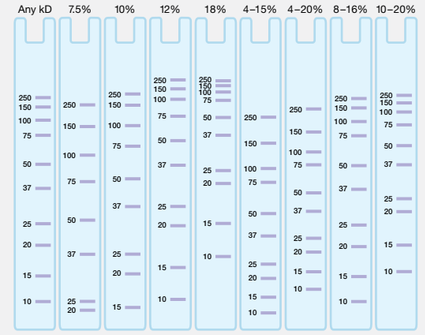
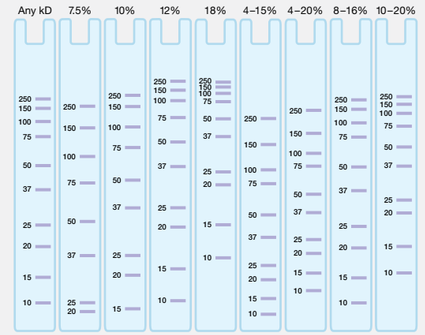
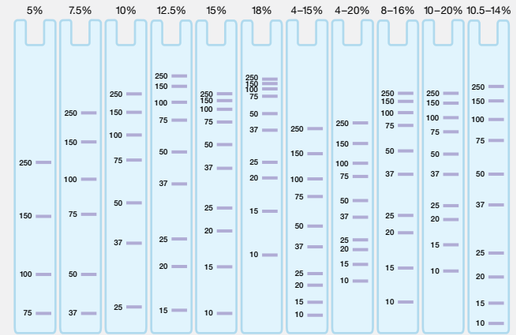
Precast Gels for Second-Dimension SDS-PAGE

Bio-Rad offers precast gels in two size formats and in a variety of formulations, some of which feature IPG wells to hold two lengths of ReadyStrip™ IPG strips (7 cm and 11 cm).

Bio-Rad's TGX™ (Tris-Glycine eXtended shelf life) precast gels are Laemmli gels with a proprietary modification that extends shelf life to 12 months and allows gels to be run at higher voltages without producing excess heat. The TGX formulation does not require special, expensive buffers. Like Tris-HCl gels, TGX gels use a discontinuous buffer system, with glycinate as the trailing ion, and are, therefore, compatible with conventional Laemmli and Tris/glycine/SDS buffers. These are the best choice when long shelf life is needed and traditional Laemmli separation patterns are desired. TGX Stain-Free™ gels are Laemmli-like extended shelf life gels that allow rapid fluorescent detection of proteins with the stain-free enabled imagers Gel Doc™ EZ and ChemiDoc™ MP, eliminating staining and destaining steps. Other precast gel formulations have also been developed to circumvent the shelf life issues of Tris-HCl systems.

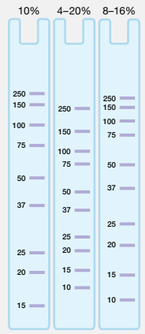
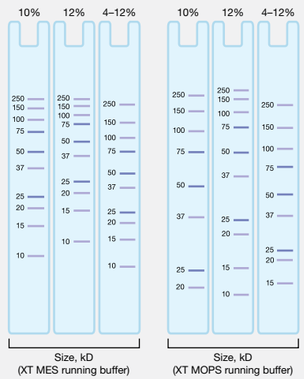
Precast Gels for Second-Dimension SDS-PAGE (contd.)

Gel Format and Formulation	Selection Criteria	Composition	Migration (%T)
Mini-PROTEAN* **(for 7 cm IPG Strips)**			
Mini-PROTEAN TGX Stain-Free	Laemmli-like extended shelf life gels Best choice when long shelf life is needed and traditional Laemmli separation patterns are desired Stain-Free formulation includes an additive for rapid fluorescence detection without staining	**Any kD** **7.5%** **10%** **12%**	
Mini-PROTEAN TGX	Laemmli-like extended shelf life gels Best choice when long shelf life is needed and traditional Laemmli separation patterns are desired	**Any kD** **7.5%** **10%** **12%** **4–15%** **4–20%**	
Mini-PROTEAN Tris-Tricine	Best choice for separation of low MW proteins	16.5% 10–20%	

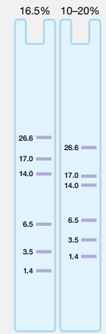
Gel Format and Formulation	Selection Criteria	Composition	Migration (%T)
Criterion* **(for 11 cm IPG Strips)**			
Criterion TGX	Laemmli-like extended shelf life gels Best choice when long shelf life is needed and traditional Laemmli separation patterns are desired	**Any kD** 7.5% 10% 12% **18%** **4–15%** **4–20%** **8–16%** **10–20%**	
Criterion TGX Stain-Free	Laemmli-like extended shelf life gels Best choice when long shelf life is needed and traditional Laemmli separation patterns are desired Stain-Free formulation includes an additive for rapid fluorescence detection without staining	**Any kD** 7.5% 10% 12% **18%** **4–15%** **4–20%** **8–16%** **10–20%**	
Criterion Tris-HCl	Reagents are easy to prepare, inexpensive, and readily available Best choice when switching between precast and handcast gels and need to compare results	5% **7.5%** **10%** **12.5%** **15%** **18%** **4–15%** **4–20%** **8–16%** **10–20%** **10.5–14%**	

* All gel percentages listed in bold are available in IPG and/or prep-well comb format.

Precast gels for Second-Dimension SDS-PAGE (contd.)

Gel Format and Formulation	Selection Criteria	Composition	Migration (%T)
Criterion* (for 11 cm IPG Strips)			
Criterion Stain Free Tris-HCl	Reagents are easy to prepare, inexpensive and readily available Best choice when switching between precast and handcast gels and when comparing results Stain-Free formulation includes an additive for rapid fluorescence detection without staining	10% 4–20% **8–16%**	
Criterion XT Bis-Tris	Offer long shelf life, but require dedicated sample and running buffers	**10%** **12%** **4–12%**	
Criterion XT Tris-Acetate	Offer best resolution of high molecular weight proteins, but require dedicated sample and running buffers	7% **3–8%**	

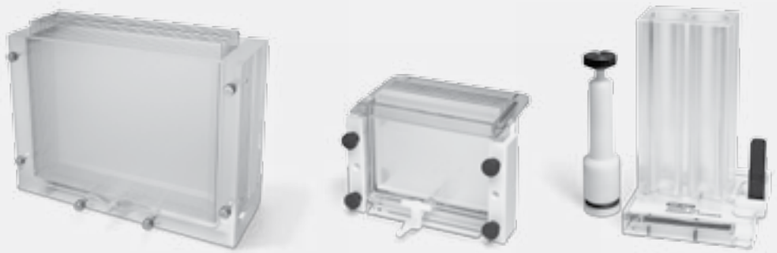
* All gel percentages listed in bold are available in IPG and/or prep-well comb format.

Gel Format and Formulation	Selection Criteria	Composition	Migration (%T)
Criterion* (for 11 cm IPG Strips)			
Criterion Tris-Tricine	Best choice for separation of low MW proteins	**16.5%** 10–20%	

Casting SDS-PAGE Gels Using Multi-Casting Chambers

In general, proteomics work requires running several IPG strips and second-dimension gels per experiment. It is important that gels have a very similar composition. The best way to ensure that handcast gels have the same composition is to cast them at the same time in a multi-casting chamber. This is especially important when casting gradient gels. Details of the assembly and use of multi-casting chambers are available in their accompanying instruction manuals. Tips that generally apply to all multi-casting systems are:

- Before assembling the casting chamber, glass plates should be carefully cleaned with Bio-Rad cleaning concentrate and thoroughly rinsed with deionized water
- Each pair of glass plates (two per gel) should be separated from the next by a spacer sheet; the spacer sheet allows easier separation of the cassettes after gel polymerization
- The volume of gel solution should be determined by measuring the volume of water needed to fill the assembled glass plates to the desired level in the multi-casting stand
- Allow overnight polymerization to compensate for the low concentrations of catalysts (recommended to ensure that polymerization does not start while the gradient gels are being cast)



Apparatus for casting multiple gels. Multi-casting chambers for 12 PROTEAN Plus™ gels or for 12 Mini-PROTEAN gels allow uniform casting of gradient gels. Gradient makers are available for both size formats.

Transition from First to Second Dimension

The transition from first- to second-dimension gel electrophoresis involves the following:

- Equilibration, which involves two steps that treat the focused IPG strips with an SDS-containing buffer to prepare the proteins for SDS-PAGE. The first equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye (optional). The second equilibration step replaces the reductant with iodoacetamide to alkylate the thiol groups. Equilibration ensures the proteins are coated with dodecyl sulfate and all cysteines are reduced and alkylated
- Embedding of the strip on the top of the second-dimension gel. The equilibrated IPG strips are placed on top of the gel and sealed in place with molten agarose solution to ensure good contact between the gel and the IPG strip

Methods for equilibrating and embedding IPG strips onto second-dimension gels are available in Part II of this guide.

Power Conditions and Reagents for SDS-PAGE

For SDS-PAGE, use running conditions that provide optimum separation across the size range of interest and that maintain the temperature of the system during operation. For a complete discussion of running conditions and the parameters that affect them, please refer to A Guide to Polyacrylamide Gel Electrophoresis and Detection, Bio-Rad bulletin 6040. For second-dimension SDS-PAGE, include a short, low voltage (50 V) step at the beginning of the run to ensure that all of the proteins are removed from the IPG strip before final voltages are applied.

Molecular Weight (MW) Estimation

SDS-PAGE is a reliable method for estimating the MW of an unknown protein. The migration rate of a protein–SDS complex is inversely proportional to the logarithm of its MW: the larger the polypeptide, the more slowly it migrates in a gel. The key to accurate MW determination is selecting separation conditions that produce a linear relationship between log (MW) and migration within the likely MW range of the unknown protein. These parameters are discussed more thoroughly in Molecular Weight Determination by SDS-PAGE (bulletin 3133), and a protocol for MW estimation is provided in Part II of this guide.

For best results, separate the protein sample on the same gel with a set of protein standards. See The Little Book of Standards (bulletin 2414) and the Protein Standards Application Guide (bulletin 2998) for more information regarding selection of protein standards. Mixtures of standard proteins with known MW can be unstained, prestained, or include tags for development with various secondary reagents (useful when blotting). Standards can be run in a reference well or attached to the end of a focused IPG strip by filter paper onto the second-dimension gel. For convenience, Bio-Rad's Precision Plus Protein standard plugs (catalog #161-0378), which are embedded in agarose plugs, can also be used.

After separation, determine the relative migration distance (R_f) of the protein standards and of the unknown protein. R_f is defined as the mobility of a protein divided by the mobility of the ion front (Figure 1). Because the ion front can be difficult to locate, mobilities are normalized to the tracking dye that migrates only slightly behind the ion front:

R_f = (distance to band)/(distance to dye front)

Using the values obtained for the protein standards, plot a graph of log (MW) vs. R_f (see below). The plot should be linear for most proteins, provided they are fully denatured and that the gel percentage is appropriate for the MW range of the sample. The standard curve is sigmoid at extreme MW values, because the sieving affect of the matrix is so large at high MW that molecules are unable to penetrate the gel; but at low MW, the sieving effect is negligible and proteins migrate almost freely. To determine the MW of the unknown protein band, interpolate the value from this graph (Figure 2).

Gradient SDS-PAGE gels can also be used to estimate MW. In this case, log (MW) is proportional to log (%T). With linear gradients, %T is proportional to the distance migrated, so the data can be plotted as log (MW) vs. log (migration distance). Standard curves are actually sigmoid. The apparent linearity of a standard curve may not cover the full MW range for a given protein mixture in a particular gel. However, log (MW) varies sufficiently slowly to allow fairly accurate MW estimates to be made by interpolation, and even extrapolation, over relatively wide ranges.

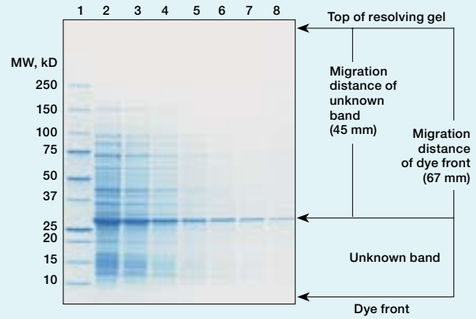


Fig. 1. Example showing MW determination of an unknown protein. Lane 1, 10 µl of Precision Plus Protein unstained standards; lanes 2–8, a dilution series of an *E. coli* lysate containing a hypothetical unknown protein (GFP). Proteins were separated by SDS-PAGE in a Criterion 4–20% Tris-HCl gel and stained with Bio-Safe Coomassie stain.

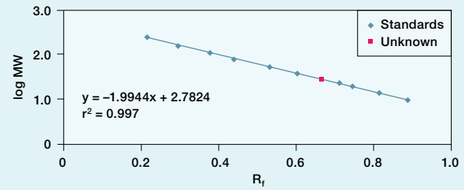


Fig. 2. Determining the MW of an unknown protein by SDS-PAGE. A standard curve of the log (MW) versus R_f was generated using the Precision Plus Protein standards from Figure 1. The strong linear relationship ($r^2 > 0.99$) between the proteins' MW and migration distance demonstrates exceptional reliability in predicting MW.

CHAPTER 5

Detection

Detection of Proteins in Gels

In 2-D electrophoresis, proteins in gels are most commonly visualized using total protein stains. Selection of the most appropriate stain involves consideration of the stain characteristics, limitations with regard to the sensitivity of detection and the types of proteins it stains best, downstream applications, and the type of imaging equipment available (see Chapter 6). For use in proteomics applications, stains should be compatible with high-throughput protocols and downstream analysis, including digestion and mass spectrometry (Patton 2000).

It is also possible to label protein samples after sample preparation and prior to IEF with fluorescent dyes such as the CyDye DIGE fluors (Westermeier and Scheibe 2008). At the time of writing, three dyes with spectrally different excitation and emission wavelengths were available, allowing labeling of up to three different samples and their separation in a single 2-D gel. The dyes are matched for size and charge to obtain migration of differently labeled identical proteins to the same spot positions. The labeled samples are mixed together before they are applied on the gel of the first dimension. After separation, the gels are scanned with fluorescence imagers at the different wavelengths.

The following are general tips for staining 2-D gels:

- 2-D gels are clearer, sharper, and more reproducible when less protein is loaded. When sample preparation and IEF conditions are not optimized, it is often beneficial to load relatively little protein and to use a relatively sensitive staining technique
- To identify low-abundance proteins, apply a high protein load and use a high-sensitivity stain (for example, silver or a fluorescent stain) (Corthals et al. 2000)
- To obtain enough protein for mass spectrometry, apply a high protein load and use a compatible staining procedure
- For quantitative comparisons, use stains with broad linear ranges of quantitation (for example, Flamingo™, Oriole™, and SYPRO Ruby)
- Since no stain is capable of staining all proteins, consider staining replicate gels with two or more different stains. Coomassie (Brilliant) Blue appears to stain the broadest spectrum of proteins. Therefore, it is instructive, especially with 2-D gels, to stain a Coomassie Blue–stained gel with silver, or to stain a fluorescently stained gel with colloidal Coomassie Blue or silver. Often, this double staining procedure reveals a few differences between the protein patterns. It is possible to stain gels first with Coomassie Blue or a fluorescent stain, then again with silver

The sensitivity achievable in staining is determined by:

- The amount of stain that binds to the proteins
- The intensity of the coloration
- The difference in color intensity between stained proteins and the residual background in the body of the gel (the signal-to-noise ratio); unbound stain molecules can be washed out of the gels without removing much stain from the proteins

No stain interacts with all proteins in a gel in precise proportion to their mass, and all stains interact differently with different proteins (Carroll et al. 2000). The only observation that seems to apply for most stains is that they interact best with proteins with a high basic amino acid content.

Coomassie Stains

Coomassie (Brilliant) Blue is the most common stain for protein detection in polyacrylamide gels. Coomassie R-250 and G-250 are fabric dyes that have been adapted to stain proteins in gels. The “R” and “G” designations indicate red and green hues, respectively. These stains generate visible protein patterns that can be analyzed using densitometric methods.

Silver Stains

Silver stains offer high sensitivity but with a low linear dynamic range (Merril et al. 1981). Often, these protocols are time-consuming and complex. Silver staining protocols have multiple steps with critical timing; for this reason, they can be insufficiently reproducible for quantitative analysis. In addition, their compatibility with mass spectrometric protein identification techniques is lower than Coomassie stains and fluorescent dyes. There are many different silver staining techniques with differing chemistries and sensitivities.

Fluorescent Stains

Fluorescent stains fulfill almost all the requirements for an ideal protein stain by offering high sensitivity, a wide linear dynamic range (up to four orders of magnitude), a simple and robust protocol, and compatibility with mass spectrometry. These sensitive stains generate little background and are easy to use.

Because fluorescent stains require specialized instrumentation for imaging, the choice of stain may be dictated by the instrumentation available. Fluorescent dyes absorb light at one wavelength and re-emit the light at another longer wavelength. Imaging instruments differ in both the type of light delivered for absorbance and the capabilities for detecting the emitted light. The simplest and least expensive systems use UV transillumination and a camera for image capture; however, not all fluorescent stains are optimally excited by UV light. Other imaging systems use laser light to scan the gel. Laser light is monochromatic, and the laser must be selected according to the absorbance properties of the dye. Not all fluorescent gel stains absorb visible light at wavelengths supplied by imaging lasers.

Fluorescent stains can be at least as sensitive as silver stains and are, therefore, subject to some of the same potential problems stemming from high sensitivity. Clean technique is essential, as any dust or dirt transferred to the surface of the gel may appear in the fluorescence image as smudges or speckles. Contaminant proteins such as keratin will also appear in the gel image if care is not taken to minimize such contamination.

All fluorescent reagents are subject to photobleaching to varying degrees. The fluorescent stains discussed in the Protein Stains sidebar are reasonably photostable and do not degrade noticeably through routine exposure to room light during a staining procedure. However, avoid exposure of the gel or staining solution to intense light and cover the staining tray with an opaque lid or foil.

Negative Stains

These rapid stains require only ~15 min for high-sensitivity staining and generate protein bands that appear as clear areas in a white background. Zinc and copper stains do not require gel fixation and proteins are thus not altered or denatured. Negative stains can be used as a quality check before transferring to a western blot or analysis by mass spectrometry, though they are not the best choice when quantitative information is desired.

Stain-Free™ Technology

This proprietary Bio-Rad technology allows protein detection in a gel both before and after transfer, as well as total protein detection on a blot when using wet PVDF membranes, without the need for application of a stain (see sidebar).

Protein Stains

Bio-Rad total protein stain selection guide.

Total Protein Stain	Detection Method	Sensitivity (Lower Limit)	Time	Comments	MS Compatible?
Coomassie Stains	**Visible**				
Coomassie (Brilliant) Blue R-250		36–47 ng	2.5 hr	Simple and consistent; requires destaining with methanol	Yes
Bio-Safe™ Coomassie G-250		8–28 ng	1–2.5 hr	Nonhazardous staining in aqueous solution; premixed	Yes
Silver Stains	**Visible**				
Silver stain (Merril et al. 1981)		0.6–1.2 ng	2 hr	Stains glycoproteins, lipoproteins, lipopolysaccharides, nucleic acids	No
Silver Stain Plus™ kit		0.6–1.2 ng	1.5 hr	Simple, robust protocol (Gottlieb and Chavko 1987)	Limited
Dodeca™ silver stain kit		0.25–0.5 ng	3 hr	Simple, robust protocol; ideal for use with Dodeca stainers (Sinha et al. 2001)	Yes
Fluorescent Stains	**Fluorescence**				
Oriole fluorescent gel stain		0.5–1 ng	1.5 hr	Rapid protocol requires no fixing or destaining; requires UV excitation	Yes
Flamingo fluorescent gel stain		0.25–0.5 ng	5 hr	Simple protocol requires no destaining; high sensitivity, broad dynamic range; excellent for laser-based scanners	Yes
SYPRO Ruby protein gel stain		1–10 ng	Overnight	Simple, robust protocol; broad dynamic range	Yes
Negative Stains	**Visible**				
Zinc stain		6–12 ng	15 min	High-contrast results; simple, fast, and reversible; compatible with elution or blotting as well (Fernandez-Patron et al. 1992)	Yes
Copper stain		6–12 ng	10 min	Single reagent; simple, fast protocol and reversible stain; compatible with elution or blotting as well (Lee et al. 1987)	Yes
Stain-Free Technology	**Stain-Free fluorescence**	8–28 ng	5 min	No separate staining steps	Yes, but tryptophan residues are modified

Coomassie Brilliant Blue R-250

Silver Stain Kit

Silver Stain Plus Kit

Oriole Fluorescent Gel Stain

Flamingo Fluorescent Gel Stain

Zinc Stain and Destain Kit

Copper Stain

(contd.)

Coomassie Stains

Coomassie R-250 staining solution is prepared for a traditional staining procedure in which gels are stained in a methanol-water-acetic acid solution of Coomassie R-250 dye. It requires ~40 ng protein per spot for detection, though absolute sensitivity and staining linearity depend on the proteins being stained.

Bio-Safe Coomassie stain is a ready-to-use, single-reagent protein stain made with Coomassie (Brilliant) Blue G-250. It offers sensitivity similar to colloidal Coomassie stains (down to 8 ng) and a rapid staining protocol. No additional reagents besides water are required.

Fluorescent Stains

Flamingo fluorescent gel stain is prepared from a dye that binds denatured protein. Normally non-fluorescent in solution, it becomes strongly fluorescent when bound to protein. There is, therefore, no need for destaining, since unbound dye in the gel is only minimally fluorescent. A prolonged fixing step is necessary to wash buffers and SDS out of the gel prior to staining, as these substances can prevent dye binding. Flamingo fluorescent gel stain is the most sensitive of the listed fluorescent stains, with sensitivity to 0.25–0.5 ng, and it can be linear over three orders of magnitude. The simple two-step staining procedure can be completed in as little as five hours.

With a primary fluorescence excitation maximum at 512 nm and a considerably weaker excitation peak at 271 nm, Flamingo fluorescent gel stain gives the most sensitive results when imaged with laser fluorescence scanning instruments equipped with green or blue laser light sources. UV transilluminator-based systems may also be used, but extended exposure times may be required and sensitivity will not be as high.

Oriole fluorescent gel stain is sensitive and, of the stains listed, it is the easiest and most rapid to use. The one-step staining process does not require fixation or destaining, allowing protein samples to be accurately visualized and quantitated in less than two hours. Since SDS is required for optimal staining, prior fixing or washing of the gel can impair staining sensitivity.

The dye in Oriole stain is excited only weakly by wavelengths longer than 400 nm and can, therefore, only be imaged using UV-based imaging systems. Oriole's limit of detection is 1 ng or less in a typical protein spot.

SYPRO Ruby was one of the original fluorescent protein gel stains, and it has a combination of high sensitivity and wide dynamic range that cannot be achieved with traditional Coomassie blue or silver stains. SYPRO Ruby has two prominent absorbance peaks, one at ~270 nm in the UV range and the other at ~460 nm in the visible range. This allows imaging with both UV transilluminator and laser-scanning systems. Detection sensitivity in SYPRO Ruby–stained gels can be as low as 1 ng. SYPRO Ruby stains most classes of proteins with little protein-to-protein variability.

The principle advantage of SYPRO Ruby is its versatility with respect to imaging requirements. It is, however, time-consuming to use and does not produce the high-quality mass spectrometric data generated with other fluorescent stains (Berkelman et al. 2009).

(contd.)

Silver Stains

Three silver staining methods are recommended for use with 2-D gels. Though they are based on slightly different chemistries, they have similar protein sensitivities.

The Bio-Rad silver stain kit, based on the method of Merril et al. (1981), can be up to 100 times more sensitive than Coomassie Blue R-250 dye staining and allows visualization of heavily glycosylated proteins in gels. Protein spots containing 10–100 ng of protein can be easily seen. Proteins in gels are fixed with alcohol and acetic acid, then oxidized in a solution of potassium dichromate in dilute nitric acid, washed with water, and treated with silver nitrate solution. Silver ions bind to the oxidized proteins and are subsequently reduced to metallic silver by treatment with alkaline formaldehyde. Color development is stopped with acetic acid when the desired staining intensity has been achieved. This method is not compatible with mass spectroscopic analysis since the oxidative step affects protein mass.

Silver Stain Plus stain from Bio-Rad, based on the method developed by Gottlieb and Chavko (1987), requires only one simultaneous staining and development step. Proteins are fixed with a solution containing methanol, acetic acid, and glycerol and then washed extensively with water. The gels are then soaked in a solution containing a silver-amine complex bound to colloidal tungstosilicic acid. Silver ions transfer from the tungstosilicic acid to the proteins in the gel by means of an ion exchange or electrophilic process. Formaldehyde in the alkaline solution reduces the silver ions to metallic silver to produce the images of protein spots. The reaction is stopped with acetic acid when the desired intensity has been achieved. Silver ions do not accumulate within the gel, so background staining is light. Since this method lacks an oxidizing step, visualization of heavily glycosylated proteins and lipoproteins can be less sensitive than with the Merril stain.

Dodeca silver stain is based on the method described by Sinha et al. (2001), in which protein-bound silver ions are chemically reduced to form visible metallic silver. This stain was developed for use with the high-throughput Dodeca stainers and can be used with mass spectrometry.

Stain-Free Technology

A special additive in Bio-Rad's Criterion Stain Free™, Criterion™ TGX Stain-Free™, and Mini-PROTEAN® TGX Stain-Free™ gels covalently modifies tryptophan residues when activated with UV light. This enhances the proteins' intrinsic fluorescence and shifts the emission into the visible range (>400 nm), allowing protein detection (with a stain-free compatible imager, such as the Gel Doc™ EZ or ChemiDoc™ MP systems) in a gel both before and after transfer, as well as total protein detection on a blot when using wet PVDF membranes.

This system is ideal for quick sample assessment during purification procedures and as a precursor to blotting and profiling workflows in which Coomassie (Brilliant) Blue staining is ordinarily used. The sensitivity of the Stain-Free system is comparable to that of staining with Coomassie Blue for proteins with a tryptophan content >1.5%; sensitivity superior to Coomassie staining is possible for proteins with a tryptophan content >3%. Proteins that do not contain tryptophan residues are not detected.

Dodeca High-Throughput Stainers

Dodeca stainers are high-throughput gel staining devices available in two sizes: the small size accommodates up to 24 Criterion gels while the large size can accommodate up to 12 large-format gels. The stainers feature a shaking rack designed to hold staining trays at an angle to allow air bubbles to escape and ensure uniform gel staining to protect gels from breaking. Use of the stainers ensures high-quality, consistent results and eliminates gel breakage from excess handling. They are compatible with the following stains:

- Bio-Safe Coomassie (Brilliant) Blue G-250 stain
- Coomassie (Brilliant) Blue R-250 stain
- SYPRO Ruby protein gel stain
- Flamingo fluorescent protein gel stain
- Oriole fluorescent gel stain
- Dodeca silver stain kits

High-Throughput Dodeca Stainers

Detection of Proteins on Western Blots

Certain synthetic membranes bind proteins tightly and can be used as supports for solid-phase immunoassays, staining, or other analysis. These membranes, known as western blots, are useful for the identification of specific proteins and protein modifications.

2-D electrophoresis can be combined with western blotting for monitoring the posttranslational modification of trace proteins in complex mixtures and evaluating the specificity of antibodies and antisera. Numerous techniques are available for the transfer of proteins to membranes and for the probing of western blots with antibodies, stains, and other reagents. These techniques are described in more detail in the Protein Blotting Guide (bulletin 2895).

CHAPTER 6

Image Acquisition, Analysis, and Spot Cutting

Finding Protein Spots of Interest

After 2-D gels are stained, the protein patterns can be digitized and analyzed with an image evaluation system comprising an imaging device and analysis software. Following analysis, spots of interest can be excised from gels for further analysis, by mass spectrometry for example (see Chapter 7).

Image Acquisition

In proteomic applications, selecting the image acquisition device depends on the staining technique used. A number of imaging systems are capable of multiple detection modes and can be used with a variety of applications.

- Densitometers enable the visualization of gels stained with visible light–absorbing stains such as Coomassie, negative, or silver stains
- Charge-coupled device (CCD) camera systems can feature different light sources for greater application flexibility. They can be used for visualization of visible and fluorescent stains and of chemiluminescence in some cases. Systems offer transillumination (visible or UV light source underneath the gel or blot) or epi-illumination (colored or white light positioned above the sample). Heat in the camera system can manifest as noise, and this noise can prevent detection of faint chemiluminescent signals above the background. Supercooled CCD cameras reduce image noise, allowing detection of faint signals
- Laser-based scanners offer the highest sensitivity, resolution, and linear dynamic range. They are powerful image acquisition tools for electrophoresis gels and blots stained with fluorescent dyes. Lasers can be matched to the excitation wavelengths of a multitude of fluorophores

Imaging Systems

System Type and Application

	GS-900™ Densitometer	ChemiDoc™ MP	Gel Doc™ EZ	PharosFX™ and PharosFX Plus
Type of imager	Densitometer	CCD camera-based	CCD camera-based	Laser-based
Light source options	Epi- and transillumination of white light	Transillumination of UV and white light* Epi-illumination by LEDs (red, green, blue, and white)	Transillumination of UV and white light	488 nm external laser 532 nm internal laser 635 nm external laser
Optimized applications				
Visible stains	•	•	•	–
UV light–excited fluorescent stains	–	•	•	–
Visible light–excited fluorescent stains and labels	–	•	–	•
Fluorescent multiplexing	–	•	–	•
Chemiluminescence	–	•	–	–
Stain-Free™	–	•	•	–

* White light conversion screen is required.

Bio-Rad's GS-900 calibrated imaging densitometer has transmittance and true reflectance capabilities that allow accurate scans of samples that are either transparent (gels and film) or opaque (blots). It provides high-quality imaging to resolve close spots and a variable resolution feature to preview and crop images.

Bio-Rad's ChemiDoc MP supercooled CCD system provides maximum flexibility. It offers transillumination of both UV light (for imaging UV fluorescent stains) and white light (for imaging visible stains). It also offers optional LED epi-illumination in red, green, and blue for single fluorescent stains or fluorescent multiplexing. In addition, it can also image stain-free gels, which require no staining or destaining and are ready for imaging in a matter of minutes after completing the SDS-PAGE run.

The PharosFX systems use multiple lasers, which enhance application flexibility and allow optimum excitation of single- or multicolor fluorescent samples to enable detection of most fluorescent dyes and labels. Computer-controlled, user-accessible filter wheels have eight filter slots, supporting multiplex or multicolor fluorescence imaging applications in gels and blots, such as Qdot multiplex blotting, DIGE, and gel staining with Pro-Q stains. The Molecular Imager® PharosFX system has all the features of the PharosFX Plus imager for fluorescence and visible detection, but it lacks the storage phosphor option for imaging radioisotopes.

Image Analysis

Following image acquisition, a robust software package is required to analyze and present the data and to draw conclusions from 2-D gel images. The software should provide a variety of tools to enhance the user's ability to evaluate the acquired data. For example, the software should be able to adjust contrast and brightness and magnify, rotate, resize, and crop gel images. It should measure total and average quantities and determine relative amounts of protein. It should also be capable of determining the presence/absence and up- or downregulation of proteins, their molecular weight, pI, and other values.

Following this initial analysis, computer-assisted image analysis software should allow:

- Storage and structuring of large amounts of collected experimental image data
- Rapid and sophisticated analysis of experimental information
- Supplementation and distribution of data among labs
- Establishment of 2-D-protein data banks

PDQuest™ 2-D Analysis Software

Bio-Rad's PDQuest 2-D analysis software is used for analyzing and creating databases for 2-D electrophoresis gels. It provides a series of "wizards" for the analysis of digitized gel images and for spot detection and quantitation, gel comparison, and statistical analysis. The Experiment Wizard guides selection of gels for analysis, detection of spots of interest, creation of an experiment, and matching of gels. The Spot Detection Wizard then guides the identification and quantitation of the spots in gel images.

After detection, gels in the same series are placed in an experiment for comparison, statistical analysis, and databasing. Histograms allow quick comparisons of the quantities of the same spot in all the gels in an experiment. Spots can also be compared qualitatively, organized into user-defined sets for further analysis, and annotated and databased for easy identification. Spots from different experimental series can be organized and compared in higher-level experiments. PDQuest can be used to simultaneously analyze thousands of spots on hundreds of gels. Data can be exported to other applications, such as spreadsheets, for further analysis.

PDQuest software has the further advantage of integration with Bio-Rad's EXQuest™ spot cutter, which accurately locates and excises protein spots from 2-D gels or blots at high speed (up to 600 spots per hour) and then loads them into 96- or 384-well microplates or 96-tube racks for downstream processing and analysis.

PDQuest has no imaging functions besides driving the camera system in the ExQuest spot cutter, but it can read and import multiple file formats from other gel imaging software packages like Quantity One®.

Image Optimization, Spot Detection, and Quantitation
Before any software can detect the protein spots of a 2-D gel, raw image data must be optimized and the gel background subtracted.

PDQuest software models protein spots mathematically as 3-D Gaussian distributions and uses the models to determine protein maxima. A 3-D Gaussian spot is a precise representation of an original scanned spot. Gaussian curves are fitted to the scanned spot in the X and Y dimensions, and then additional modeling is performed to create the final Gaussian spot. Using Gaussian modeling, it is possible to accurately quantitate overlapping spots, spots in gel streaks, and multiple spots in dense clusters.

The accuracy of automatic spot detection depends on the quality of the 2-D gels and their images. Correction capabilities of PDQuest software can be used to add undetected spots to the list of spots or to delete spots that arise from gel artifacts.

Gel Comparison
The next step in 2-D gel evaluation is identification of proteins that are present in all gels of a series. Since inherent problems with gel-to-gel reproducibility affect the positions of spots within a gel series, gel analysis software must be able to detect minor shifts in individual spot position within the gel series.

Many software packages for automatic gel comparison are created with the assumption that the relative positions of spots are altered only slightly relative to each other, and they allocate the spots on this basis. Prior to automatic gel comparison, PDQuest software selects the best 2-D gel of a gel series as a reference, or standard gel, and compares all other 2-D gels to this gel. Proteins in a gel series that are not present in the reference gel can be added automatically so that the reference gel includes all proteins of a gel series.

PDQuest includes the ability to match spots with no manual assistance, and it is possible to display up to 100 enlarged details of 2-D gels on the screen simultaneously, enabling rapid and error-free determination of the matching quality.

Data Normalization
When comparing gels in an experiment, there is often some variation in spot size and intensity among gels that is not due to differential protein expression. Multiple normalization methods can be used to compensate for gel-to-gel variations in spot intensity caused by inconsistencies in sample loading, gel staining, and imaging. To accurately compare spot quantities among gels, compensation for these variations in spot intensity, which are not related to expression levels, is required.

Data Analysis and Reporting
With PDQuest software, all gels in a single experiment are viewed as a unit. To compare gels from different experiments, the reference images are compared. In such comparisons, each spot is automatically assigned a number such that identical spots have identical numbers. In an experiment, the molecular weight and pI values for known protein spots can also be entered. With these data, PDQuest can estimate molecular weight and pI values for all the spots in the experiment.

Analysis sets allow the study of sets of proteins that are statistically and scientifically significant and to identify spots to cut using the ExQuest spot cutter. There are six different kinds of analysis sets:

- **Qualitative analysis sets** — spots that are present in one gel but not in another
- **Quantitative analysis sets** — spots whose intensity (amount) has increased or decreased by a certain degree, or whose intensity has changed above, below, or within the fold change factor that you specify
- **Statistical analysis sets** — spots that are significant according to the statistical test that you apply
- **Arbitrary analysis sets** — manually selected spots
- **Boolean analysis sets** — created by comparing two or more analysis sets (for example, set C could be made up of those spots present in both sets A and B)
- **Matching analysis sets** — spots that are either unique to one member or present in all members

Once proteins of interest are determined, the corresponding analysis sets are uploaded to the spot cutter. The spots of interest are then excised from the gels and digested to release peptides for analysis by various mass spectrometry methods.

Spot Cutting from 2-D Gels

Spots of interest can be excised from gels either manually (for example, with a scalpel, razor blade, or modified pipet tip) or with an automated spot cutting system. The advantages of automated systems are numerous and include improved precision and reproducibility, tracking of gel spots, and decreased risk of contamination. The excised gel plugs are then transferred to microplates or other vessels for digestion and further analysis.

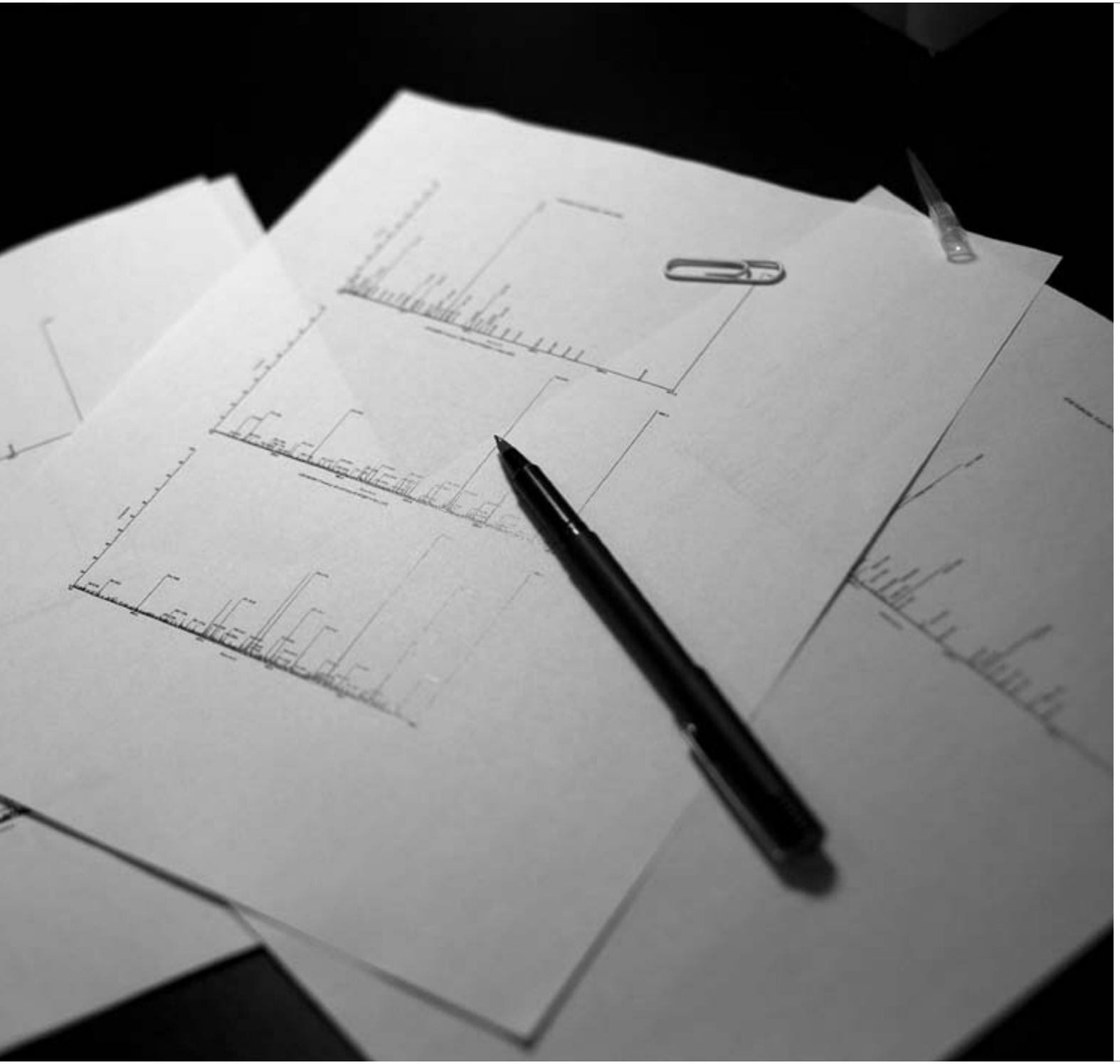
EXQuest Spot Cutter

Bio-Rad's EXQuest spot cutter accurately locates and excises protein bands or spots from gels or blots and loads them into 96- or 384-well microplates or 96-tube racks for downstream processing and analysis. Its camera works with PDQuest 2-D analysis software to visualize gels and blots that are either visibly or fluorescently stained. In 2-D electrophoresis applications, PDQuest software tracks the protein bands or spots through spot cutting and protein identification, which is usually accomplished using mass spectrometry.

The EXQuest spot cutter allows use of any common proteome separation and staining methods:

- Freestanding or plastic- or glass-backed 2-D and 1-D SDS-PAGE gels
- PVDF and nitrocellulose membrane blots
- Gels or membranes stained for proteins with visible stains (such as silver and Coomassie blue stains) or fluorescent stains (such as Flamingo™, Oriole™, and SYPRO Ruby protein stains)

ExQuest Spot Cutter



CHAPTER 7

Identification and Characterization of 2-D Protein Spots

Beyond Excision

2-D electrophoresis has the unique capability of simultaneously displaying several hundred proteins. When coupled with the ability of mass spectrometry to identify and characterize small quantities of protein, 2-D electrophoresis is a very powerful and effective analytical method.

Several mass spectrometric techniques can be used for protein identification at the end of a 2-D electrophoresis workflow. Most of these methods first require proteolytic digestion of the protein into discrete fragments that can be eluted from the excised gel plug. The most basic mass spectrometric method, peptide mass fingerprinting, simply determines accurate masses of the peptides generated. These masses are then compared to a database, and the protein of origin can often be uniquely identified. Another technique, tandem mass spectrometry (MS/MS) further fragments selected peptides along the peptide backbone, allowing the generation of limited sequence information that can be used to refine the protein identification step.

Proteolytic Digestion

In-gel digestion (Rosenfeld 1992) of selected proteins is part of the sample preparation process for mass spectrometry, and it comprises four basic steps: destaining (washing) the gel pieces, reduction and alkylation, proteolytic cleavage of the protein, and extraction of the resultant peptides.

Washing

After excision of the protein spot of interest from the gel, most protocols require destaining of the proteins before proceeding. The destaining or wash protocol depends on the stain used for visualization. Commonly used protocols for various stains are described in Part II of this guide.

Reduction and Alkylation

Reduction and alkylation together reduce and irreversibly block the formation of inter- and intramolecular disulfide bridges, which can significantly improve the efficacy of proteolytic cleavage and subsequent mass spectrometry.

Proteins excised from 2-D gels have usually been reduced and alkylated either during sample preparation or equilibration prior to the second dimension and may not require this step. This step is mandatory if upstream processing did not incorporate reduction and alkylation. Any reduction or reduction plus alkylation step must be followed by a cleanup step prior to mass spectrometry.

In-Gel Proteolytic Digestion

Proteolytic digestion can be performed directly on processed gel pieces. Because proteases are also subject to autolysis, always include a blank piece as a control. Proteases used for this purpose are selected for their efficiency in in-gel digestion and for their defined cleavage specificity, which allows prediction of the generated peptide masses. The most commonly used protease is trypsin, but other proteases used include LysC, GluC, ArgC, AspN, and LysN, which cleave to either the C- or N-terminal side of a single amino acid, as signified by their nomenclature. These enzymes are all commercially available as preparations that have been specifically modified for use prior to mass spectrometry. Enzymes specifically recommended for mass spectrometry should always be used for in-gel digestion.

- Use trypsin (modified porcine pancreatic trypsin, mass spectrometry grade) for initial protein digestion. Trypsin is one of the most specific proteases and cleaves at the C-terminal side of Arg and Lys
- Use GluC, AspN, or LysC with proteins of smaller mass. These enzymes generate fewer peptides of larger mass than trypsin, which may generate fragments too small for definitive identification
- Use acid hydrolysis, cyanogen bromide cleavage, or other chemical methods if alternatives to enzymatic digestion are required
- Some proteins are processed forms of larger proteins; therefore, once the protein is identified based on a trypsin digestion, other methods can be used to define the N- and C-termini of the fragment

The resulting peptides can be extracted with acetonitrile, dried under vacuum, and dissolved in a small amount of water. Prior to mass spectrometry, the samples should be further purified by solid phase extraction, for example using ZipTip pipet tips. A protocol is provided in Part II of this guide.

Identification by Mass Spectrometry

Identification of the peptides derived from digestion can be achieved using several mass spectrometry techniques. Only a brief overview of mass spectrometry theory and techniques is presented here. Refer to the literature from mass spectrometer vendors for more information about systems and methods.

Mass spectrometry systems contain the following components (Figure 7.1):

- **Ionization source** — converts the sample into gas-phase ions, which are then injected into a mass analyzer. The two ionization sources most commonly used for peptide mass spectrometry are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI)
  - **MALDI** — the protein is mixed with an organic molecule (the “matrix”), deposited onto a planar substrate, allowed to dry, and illuminated with a pulsed UV laser. The matrix compound absorbs the laser energy and promotes peptide ionization, typically generating singly-charged molecular ions. MALDI is useful for high-throughput applications but is limited by ion suppression (particularly in complex peptide mixtures) and chemical noise from the matrix in the low mass range
  - **ESI** — a flowing liquid is passed through a charged orifice to produce charged droplets, which are then desolvated to yield gas-phase peptide ions. ESI can be coupled directly to liquid-phase separations such as chromatography (LC-MS) and generates multiply-charged molecular ions that bring mass-to-charge ratio (m/z) values within the mass range of mass spectrometry instruments most commonly used with ESI
- **Mass analyzer** — sorts the ions according the m/z. A number of different types of mass analyzers are available, including time-of-flight (TOF), quadrupole, and ion trap systems as well as combinations of these (hydrid mass spectrometers)
- **Ion detector** — records the ion current, amplifies it, and sends it to the data analysis system where it is presented in the form of a mass spectrum. The m/z values of the ions are plotted against their intensities to show the number of components in the sample, the molecular mass of each component, and the relative abundance of the various components in the sample

The data from the mass analyzer(s) are used for protein identification, and two options are most common in the 2-D electrophoresis workflow: peptide mass fingerprinting and tandem mass spectrometry.

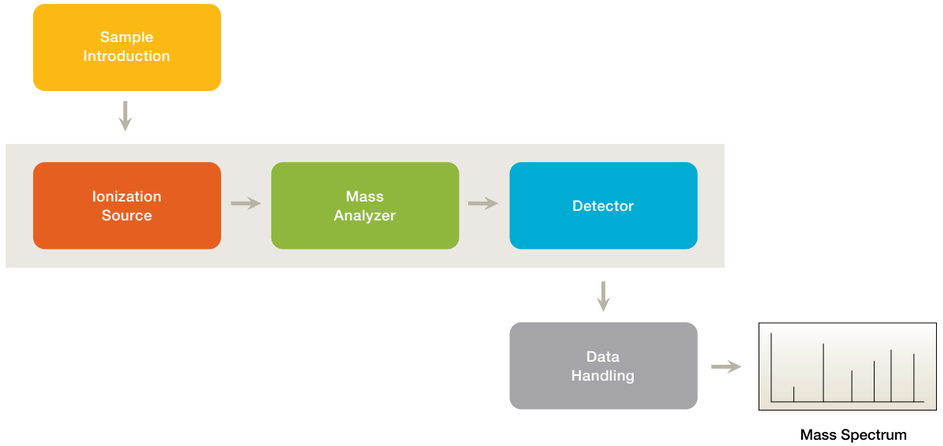


Fig. 7.1. Components of a mass spectrometer.

Peptide Mass Fingerprinting
In this method, the peptides resulting from digestion of the protein of interest are analyzed by mass spectrometry and compared to a database of calculated peptide masses generated by "in silico" cleavage of protein sequences using the same specificity as the enzyme that was employed in the experiment. Identifications ("hits") are scored in terms of confidence of match (Figure 7.2).

This approach requires simple mixtures of proteins or pure proteins and is, therefore, suitable for analysis of proteins isolated from 2-D electrophoresis. Limitations of peptide mass fingerprinting, however, include the following: (i) the protein sequence has to be present in the database of interest, and (ii) several peptides are required to uniquely identify a protein. Additionally, most algorithms assume that the peptides come from a single protein, which is why resolution in the 2-D separation is so critical. If this information does not allow unequivocal identification of the protein, peptides can then be analyzed by tandem mass spectrometry.

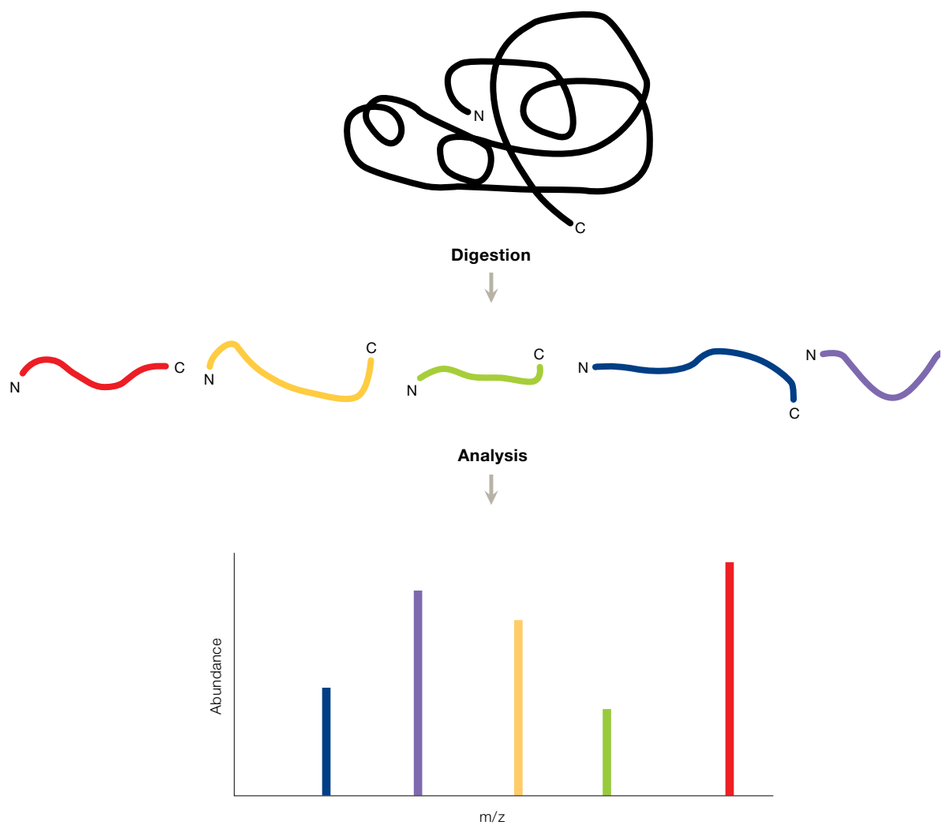


Fig. 7.2. Peptide mass fingerprinting. Peptides resulting from digestion are analyzed by mass spectrometry, and the resulting m/z values and mass spectrum are compared to theoretical values derived from "in silico" digestion of known proteins in a database.

Tandem Mass Spectrometry (MS/MS)
In MS/MS, a peptide ion is isolated in the mass analyzer and subjected to dissociation to product ion fragments. Peptides dissociate according to certain rules. For example, fragmentation typically occurs along the peptide backbone; each residue of the peptide chain is successively cut off, both in the N->C (a-, b-, c- ions) and C->N (x-, y-, z- ions) directions. The product ions resulting from the fragmentation are analyzed in a second stage of mass analysis, which enables sequence derivation (Figure 7.3). Tandem MS can allow identification of proteins from a single peptide (Lovric 2011).

Establishment of 2-D Databases

After the spots are cut, analyzed, and identified, by MS for example, the information can be imported back into the experiment as annotations. Annotations are organized in categories, for example by protein name, protein family amino acid composition, protein function, cellular location, binding properties, and translational regulation. A single spot may be annotated in multiple categories, depending on the amount and type of information available about it. Most categories contain simple text annotations. Specialized categories can be used to link spots to Internet protein databases or to open files in other applications.

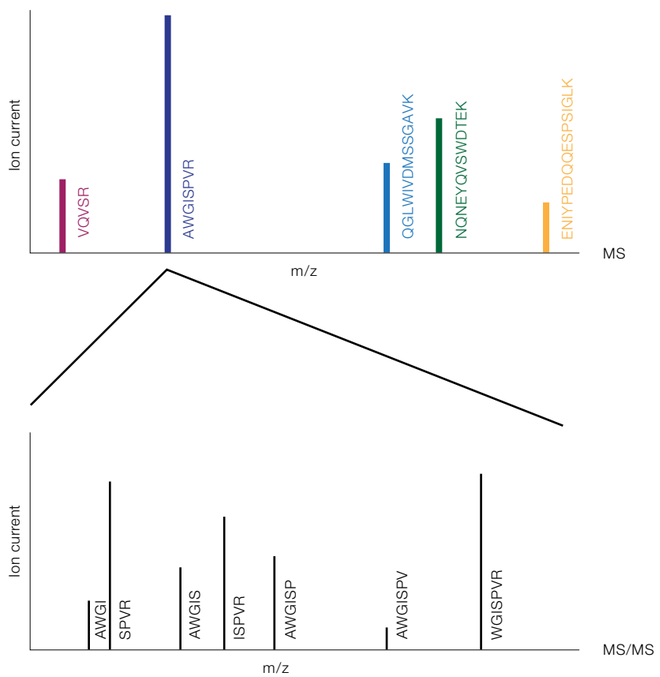
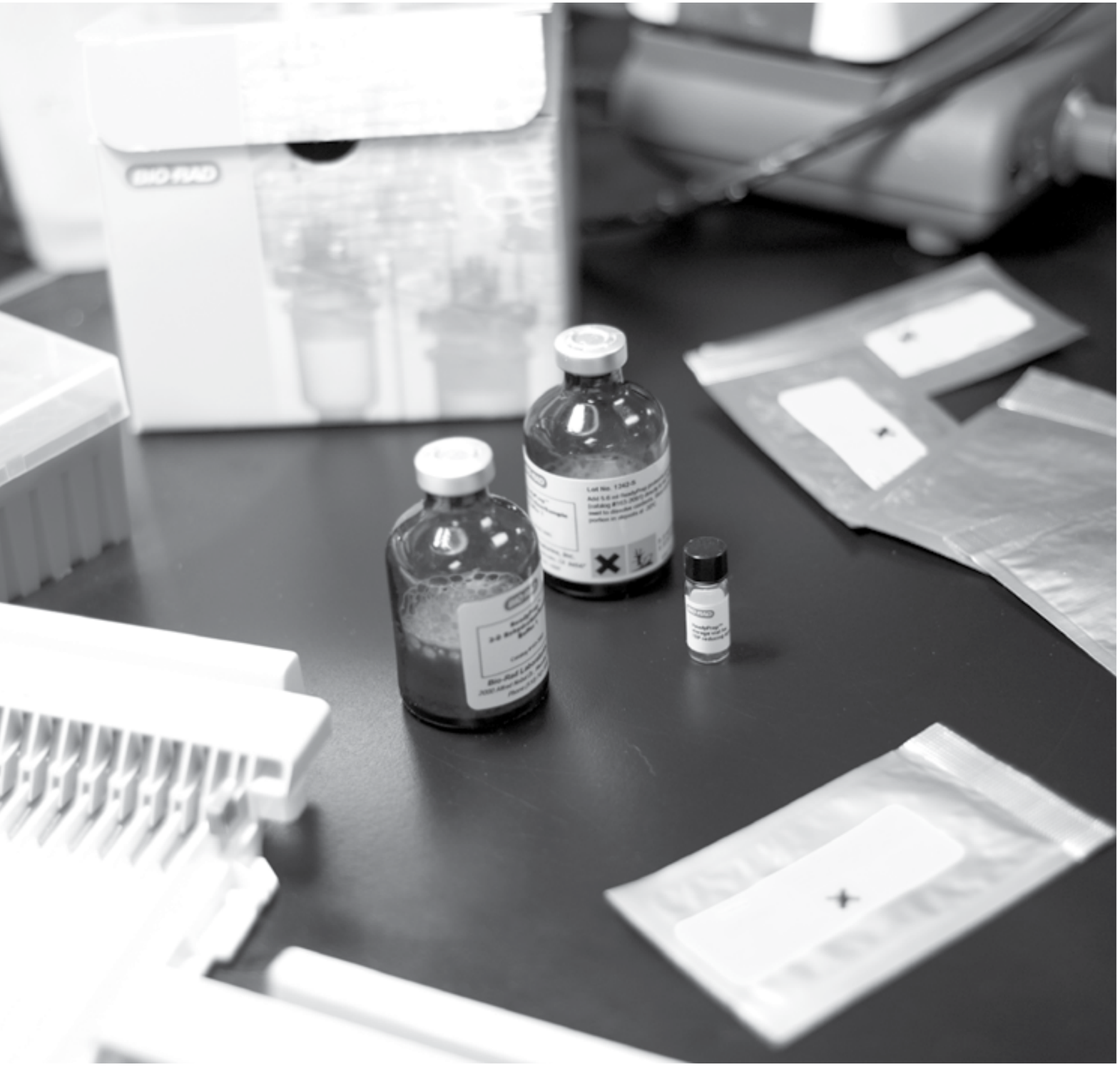


Fig. 7.3. MS/MS analysis. The first mass analyzer selects ions of a particular m/z for fragmentation. The second mass analyzer produces the mass spectrum for those fragments.



PART II
Methods

CHAPTER 8
Sample Preparation

Tips for Sample Preparation

Keep the sample preparation workflow simple (increasing the number of sample handling steps may increase variability).

Lysis (Cell Disruption)

- For each 10 mg (fresh weight) pelleted cells or animal tissue, use about 1 ml of 2-D sample solution for a protein concentration of 1–3 mg/ml. When disrupted in liquid nitrogen, samples such as liver biopsies and plant leaves contain 10–30% and 1–2% extractable protein, respectively
- To diminish endogenous enzymatic activity:
  - Disrupt the sample or place freshly disrupted samples in solutions containing strong denaturing agents such as 7–9 M urea, 2 M thiourea, or 2% SDS. In this environment, enzymatic activity is often negligible
  - Perform cell disruption at low temperatures to diminish enzymatic activity
  - Lyse samples at pH >9 using either sodium carbonate or Tris as a base in the lysis solution (proteases are often least active at basic pH)
  - Add a chemical protease inhibitor to the lysis buffer. Examples include phenylmethylsulfonyl fluoride (PMSF), aminoethyl-benzene sulfonyl fluoride (AEBSF), tosyl lysine chloromethylketone (TLCK), tosyl phenyl chloromethyletone (TPCK), ethylenediaminetetraacetic acid (EDTA), benzamidine, and peptide protease inhibitors (for example, leupeptin, pepstatin, aprotinin, bestatin). For best results, use a combination of inhibitors in a protease inhibitor cocktail
  - If protein phosphorylation is to be studied, include phosphatase inhibitors such as fluoride and vanadate
- When working with a new sample, use at least two different cell disruption protocols and compare the protein yield (by protein assay) and qualitative protein content (by SDS-PAGE)
- Optimize the power settings of mechanical rupture systems and incubation times for all lysis approaches. Mechanical cell lysis usually generates heat, so employ cooling where required to avoid overheating of the sample
- Following cell disruption, check the efficacy of cell wall disruption by light microscopy and centrifuge all extracts extensively (20,000 × g for 15 min at 15°C) to remove any insoluble material; solid particles may block the pores of the electrophoresis gel
- Direct application of clarified lysate to IPG strips is appropriate only for samples with high protein content and minimal interfering substances. Preparation of many sample types (for example, plant tissues and dilute bodily fluids) should incorporate a precipitation step to remove interfering substances and allow application of a more concentrated sample

Protein Solubilization

- Prepare fresh sample solubilization solutions daily or store them frozen in aliquots, preferably at –80°C; always use high-quality reagents and proteomics-grade water. Use urea stock solutions soon after they are made, or treat them with a mixed-bed ion exchange resin to avoid protein carbamylation by cyanate, which forms in old urea. If solutions are prepared in advance and stored, it is best to prepare them without reductant (DTT) and add the reductant directly before use
- Dissolve pelleted protein samples in 1× 2-D sample solution
- Perform a protein quantitation assay to determine the amount of total protein in each sample. Use a protein assay that is tolerant to chemicals in your samples. For samples in 2-D sample solution, for example, use the *RC DC*™ protein assay, which can tolerate up to 2% detergent
- Dilute or concentrate samples as needed to yield a final protein concentration of 1–5 mg/ml. Make dilutions in 2-D sample solution and concentrate the sample using the ReadyPrep™ 2-D cleanup kit
- Use protein extracts immediately or aliquot them into appropriately sized batches and store them at –80°C to avoid freeze-thaw cycles
- Highly viscous samples likely have a very high DNA or carbohydrate content. Fragment DNA with ultrasound during protein solubilization or by adding endonucleases like benzonase. Use protein precipitation (for example, with the ReadyPrep 2-D cleanup kit) to diminish carbohydrate content
- Do not heat samples containing urea and thiourea above 35°C as this can lead to protein modification

Buffers and Solutions

2-D sample solution (50 ml)

7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTT, 0.2% (w/v) ampholytes (pH 3–10)

Urea/thiourea stock solution	48 ml
CHAPS	2.0 g
Bio-Lyte® ampholytes, pH 3–10	250 µl
DTT	0.31 g
Bromophenol blue (1%)	10 µl
Distilled or deionized H_2O	to 50 ml

- 2-D sample solution is used for sample application and IPG strip rehydration. Bio-Rad offers various types of 2-D sample buffers, which differ in solubilizing power (see Ordering Information)
- For pH control, Tris base may be added to the 2-D sample solution at 10–40 mM. Addition of Tris increases the conductivity of the sample solution and extends the time required to focus the IPG strips
- Ampholytes are added to all IPG rehydration and sample solubilization solutions to maintain solubility of the proteins. The choice of ampholytes depends on the pH range of the IPG strip. Higher concentrations (up to 1% (w/v)) may be used, but they result in lower IEF voltage and correspondingly longer focusing times

Urea/thiourea stock solution (50 ml)

Urea	22 g
Thiourea	8 g
Distilled or deionized H_2O	to 50 ml

Filter through Whatman No. 1 paper using a Buchner funnel

Store at –80°C

1% Bromophenol blue (10 ml)

Bromophenol blue will not dissolve in unbuffered water. Prepare 10 ml of 50 mM Tris base by dissolving 60.6 mg of Tris in 10 ml of water. Add 100 mg of bromophenol blue and vortex until dissolved.

Store at 25°C.

Cell washing buffer (1 L)

10 mM Tris-HCl, pH 7.0, 250 mM sucrose

Tris base	1.21 g
Sucrose	85.58 g
Distilled or deionized H_2O	800 ml
Dissolve	
Adjust pH to 7.0 with HCl	
Distilled or deionized H_2O	to 1 L

Store at 4°C

Protein precipitation solution (100 ml)

20% (w/v) trichloroacetic acid (TCA), 0.2% DTT (w/v) in ice-cold acetone (–20°C)

Trichloroacetic acid	20 g
DTT	0.2 g
Acetone	80 ml
Dissolve	
Acetone	to 100 ml

Store at –20°C

Wash solution (100 ml)

0.2% DTT in ice-cold acetone (–20°C)

DTT	0.2 g
Acetone	80 ml
Dissolve	
Acetone	to 100 ml

Store at –20°C

SDS sample solubilization buffer (50 ml)

1% (w/v) SDS, 100 mM Tris-HCl (pH 9.5)

SDS	0.5 g
Tris base	0.6 g
Distilled or deionized H_2O	40 ml
Titrate to pH 9.5 with diluted HCl	
Distilled or deionized H_2O	to 50 ml

Store at 25°C

Cell Lysis and Protein Extraction Procedures

Suspension Cultured Human Cells

Use the MicroRotofor™ cell lysis kit (mammalian) or the protocol below, which uses 2-D sample solution and a sonicator for cell lysis and protein extraction. Use 0.5 ml of 2-D sample solution with 3 × 10^7 cells.

Reagents

- 2-D sample solution
- Cell washing buffer

Protocol

1. Pellet the cells by centrifugation at 2,000 × g for 5 min at 4°C.
2. Discard the supernatant and wash pelleted cells in cold cell washing buffer. Repeat steps 1 and 2 two times.
3. Add 2-D sample solution to the pelleted cells and suspend the pellet with a pipet.
4. Place the cell suspension on ice, incubate 5 min, and sonicate at appropriate intervals. Check lysis efficacy by light microscopy.
5. Centrifuge cell debris at 14,000 × g for 15 min and transfer supernatant to a new vial.
6. Perform a protein assay of the supernatant. A protein concentration of 3–5 mg/ml is best for 2-D electrophoresis.

Monolayer Cultured Human Cells

Use the MicroRotofor cell lysis kit (mammalian) or the protocol below, which uses 2-D sample solution and a sonicator for cell lysis and protein extraction. Use 0.5 ml of 2-D sample solution with 3 × 10^7 cells.

Reagents

- 2-D sample solution
- Cell washing buffer

Protocol

1. Carefully remove (decant) culture medium from cells. Wash cells twice with cell washing buffer.
2. Add 2-D sample solution to the cells and keep on ice for 5 min. Swirl the plate occasionally to spread the buffer around the plate.
3. Use a cell scraper to collect the lysate and transfer to a microcentrifuge tube.
4. Place the cell suspension on ice, incubate 5 min, and sonicate at appropriate intervals. Check lysis efficacy by light microscopy.
5. Centrifuge the cell debris at 14,000 × g for 15 min and transfer the supernatant to a new vial.
6. Perform a protein assay of the supernatant. A protein concentration of 3–5 mg/ml is best for 2-D electrophoresis.

Mammalian Tissue

Use the MicroRotofor cell lysis kit (mammalian) or the protocol below, which involves freezing tissue samples (for example, biopsy samples) in liquid nitrogen. Use liquid nitrogen and a mortar and pestle to grind the samples while they are still frozen. Break up any larger pieces beforehand (for example, wrap the frozen tissue sample in aluminum foil and crush with a hammer).

Reagents

- 2-D sample solution

Protocol

1. Chill a mortar with liquid nitrogen, then grind small tissue pieces in the presence of liquid nitrogen to a fine powder.
2. Immediately after grinding, transfer 60 mg tissue powder to a microcentrifuge tube containing 1.0 ml of 2-D sample solution.
3. **Optional:** sonicate the sample on ice 5 times, for 2 sec each time. Pause between sonication steps to avoid overheating.
4. Incubate the sample at room temperature for 30 min. Vortex from time to time.
5. Centrifuge at 35,000 × g for 30 min at room temperature.
6. Perform a protein assay to determine the protein concentration of the supernatant, which should be 5–10 mg/ml.
7. Dilute the supernatant with 2-D sample solution and incubate for 20 min at room temperature.

Microbial Cultures

Reproducible sample preparation from bacteria and yeast is challenging because the cells may release proteases and other enzymes into the growth medium (Harder et al. 1999, Drews et al. 2004, Poetsch and Wolters 2008). Wash the cultures thoroughly with isotonic buffers and take precautions to inactivate the proteolytic activity after cell lysis. Extensive disruption of microbial cells is required and is usually performed with the help of a French press, bead impact instruments, or sonicator.

Use the MicroRotofor cell lysis kit (bacteria), the MicroRotofor cell lysis kit (yeast), or the protocol below. This protocol relies on cell lysis with ultrasonic waves in combination with a solubilization in SDS under elevated temperature to ensure deactivation and denaturation of proteases.

Reagents

- SDS sample solubilization buffer
- 2-D sample solution
- Cell washing buffer

Protocol

1. Centrifuge cells (~5 × 10^7) at 5,000 × g for 3 min and resuspend the pellet in an equal volume of 2-D cell washing buffer heated at 37°C and centrifuge again. Repeat two more times to remove all interfering material (extracellular proteases and growth media).
2. Add ~150 µl hot (95°C) SDS sample solubilization buffer to the pellet and vortex thoroughly.
3. Sonicate the sample solution 10 times for 1 sec each at ~60 W and ~20 kHz.
4. Incubate the sample at 95°C for 5 min.
5. Cool the sample to 20°C and dilute with ~500 µl of 2-D sample solution. Incubate for another 20 min at room temperature. The final SDS concentration should not exceed 0.25% in the extract to be applied onto the IPG strip; therefore, be sure that the total volume is maintained during the SDS boiling step.
6. Centrifuge the sample solution at 20°C for 30 min at 14,000 × g and harvest the supernatant.
7. Perform the protein assay. The protein concentration should be ~5 µg/µl.

Cell Lysis and Protein Extraction Procedures (contd.)

Plant Leaves

Plant leaf cells contain reactive compounds (such as proteases, phenol oxidases, organic acids, phenols, and terpenes). To minimize the deleterious effects of these compounds on protein integrity, use the MicroRotofor cell lysis kit (plant) or follow this protocol, which involves grinding the tissue in a mortar and pestle with liquid nitrogen. Precipitate the proteins with 20% trichloroacetic acid (TCA) in prechilled acetone (–20°C). To remove the plant phenols, rinse the pellet at least twice with cold acetone (–20°C) and air-dry samples in a vacuum (Damerval 1986).

Reagents

- Protein precipitation solution
- Wash solution
- 2-D sample solution

Protocol

1. Cool protein precipitation and wash solutions to –20°C and chill a mortar with liquid nitrogen.
2. Place leaves in the mortar, add liquid nitrogen, and grind the leaves in the liquid nitrogen to a fine powder.
3. Transfer leaf powder into 20 ml protein precipitation solution and incubate for 1 hr at –20°C. Stir solution occasionally.
4. Centrifuge the solution at –20°C for 15 min at 35,000 × g.
5. Discard the supernatant, add wash solution, and suspend the pellet. Incubate for 15 min at –20°C and stir the solution occasionally.
6. Repeat steps 4 and 5 until the wash solution turns from dark to light green.
7. Centrifuge the solution at –20°C for 15 min at 35,000 × g and discard the supernatant.
8. Add 2 ml of wash solution and suspend the pellet.
9. Transfer the suspension into a shallow ceramic shell and cover with perforated Parafilm wrap.
10. Put the shell into a dessicator and apply a vacuum until the pellet (acetone powder) is dry.
11. Mix 5 mg of sample powder with ~0.5 ml of 2-D sample solution and incubate for 30 min at room temperature. Vortex from time to time.
12. Centrifuge the solution at room temperature for 15 min at >16,000 × g.
13. Collect the supernatant and perform the protein assay.

Sample Cleanup

Prior to IEF, remove contaminating salts, buffers, and other chemicals from samples by dialysis, precipitation, or buffer exchange. A protocol for buffer exchange using Bio-Rad's Micro Bio-Spin™ P-6 columns is provided here. Another alternative is the use of the ReadyPrep 2-D cleanup kit to effectively precipitate sample protein and remove contaminants. It has the additional benefit of concentrating the sample to a desired volume.

Buffer Exchange (Desalting)

Bio-Rad's Micro Bio-Spin columns are suitable for use with 1.5 or 2.0 ml microcentrifuge tubes and are completely autoclavable. They accommodate volumes of 20–75 µl; volumes less than 20 µl may affect recovery. The gel in the Micro Bio-Spin columns is suspended in either SSC buffer, pH 7.0, or Tris-HCl buffer, pH 7.4. For 2-D electrophoresis, it is best to exchange the sample into the 2-D sample solution (7 M urea, 2 M thiourea, 4% CHAPS) using the following protocol. DTT and ampholytes are added after the buffer exchange procedure.

Protocol

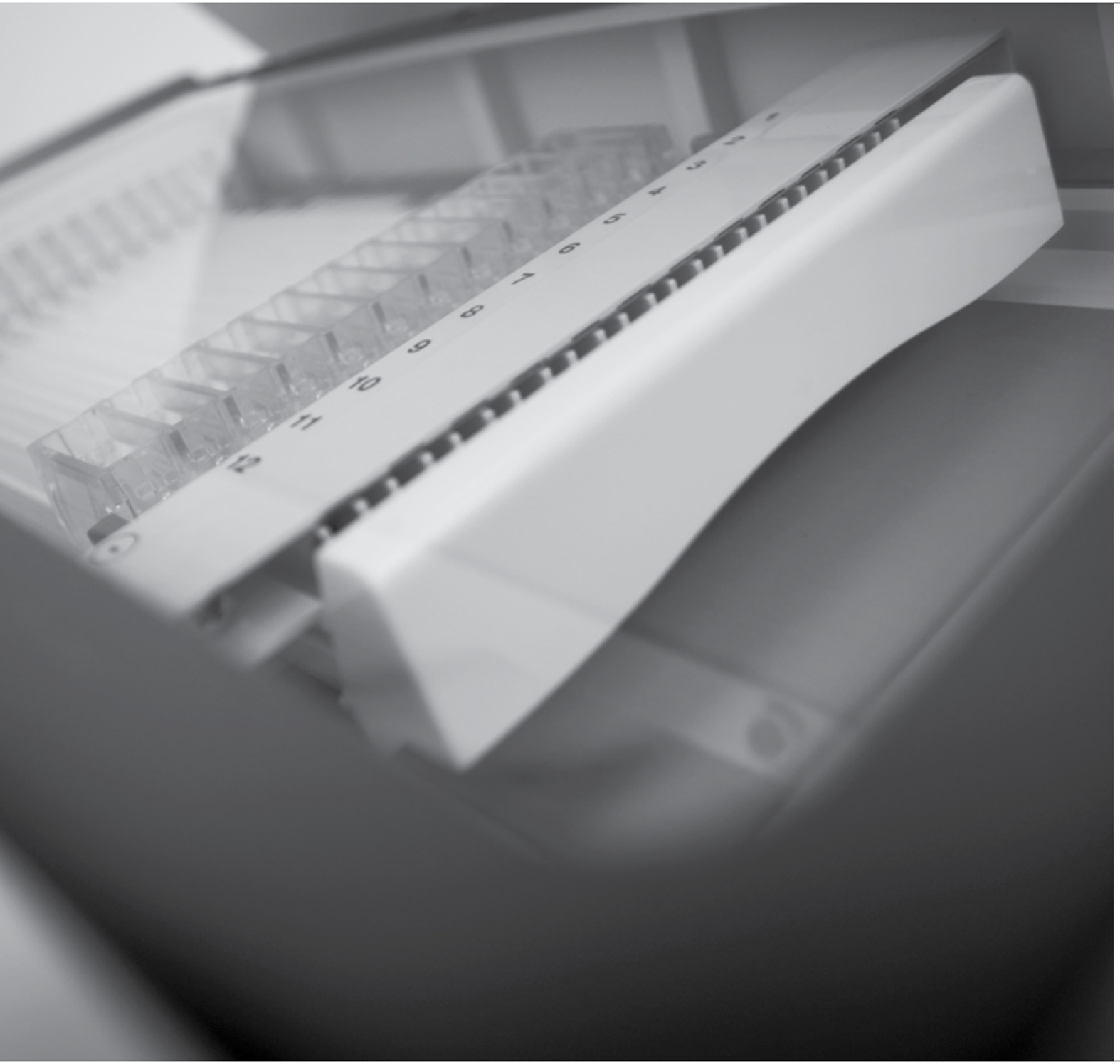
1. Invert the column sharply several times to resuspend the settled gel and remove any bubbles. Snap off the tip and place the column in a 2.0 ml microcentrifuge tube (included). Remove the top cap. If the column does not begin to flow, push the cap back on the column and then remove it again to start the flow. Allow the excess packing buffer to drain by gravity to the top of the gel bed (about 2 min). Discard the drained buffer, then place the column back into the 2.0 ml tube.
2. Centrifuge for 2 min in a microcentrifuge at 1,000 × g to remove the remaining packing buffer. Discard the buffer.
3. Apply the new buffer in 500 µl aliquots. After each application, let the buffer drain out by gravity, then centrifuge the column at 1,000 × g for 1 min to remove the buffer. Discard the buffer from the collection tube. Repeat as required. Three washes result in >99% of the buffer exchanged. Four washes result in >99.9% of the buffer exchanged.
4. Place the column in a clean 1.5 or 2.0 ml microcentrifuge tube. Carefully apply the sample (20–75 µl) directly to the center of the column. Application of more or less than the recommended sample volume may decrease column performance.
5. Centrifuge the column for 2–4 min at 1,000 × g. Following centrifugation, the purified sample is in the new buffer. Molecules smaller than the column's exclusion limit are retained by the column.

Sample Quantitation (*RC DC* Protein Assay)

The *RC DC* protein assay is based on a modification of the Lowry protocol (Lowry et al. 1951) and is both reducing agent compatible (RC) and detergent compatible (DC). Protein quantitation can be performed in complex mixtures including 2-D sample solution. It involves addition of detection reagents to a protein solution and subsequent measurement of absorbance at 750 nm with a spectrophotometer. Comparison to a standard curve provides a relative measurement of protein concentration.

Microfuge Tube Assay Protocol (1.5 ml)

1. Add 5 µl of *DC* Reagent S to each 250 µl of *DC* Reagent A needed. This solution is referred to as Reagent A′. Each standard or sample assayed requires 127 µl Reagent A′.
2. Prepare 3–5 dilutions of a protein standard (0.2–1.5 mg/ml protein). Use distilled or deionized water as the diluent.
3. Pipet 25 µl of protein standard or sample into clean 1.5 ml microcentrifuge tubes. Add 125 µl of *RC* Reagent I into each tube and vortex. Incubate the tubes for 1 min at room temperature.
4. Add 125 µl of *RC* Reagent II into each tube and vortex. Centrifuge the tubes at 15,000 x g for 5 min. Position the tubes with the cap hinge facing outward.
5. Remove the tubes as soon as centrifugation is complete. A small pellet should be visible on the hinge side of the tube. Decant the supernatant. Reposition the tubes as before. Briefly centrifuge again to bring any remaining liquid to the bottom of the tube. Use a micropipet to remove the remaining liquid.
6. Add 127 µl of Reagent A′ to each tube and vortex. Incubate tubes at room temperature for 5 min, or until the precipitate is dissolved. Vortex.
7. Add 1 ml of *DC* Reagent B to each tube and vortex immediately. Incubate at room temperature for at least 15 min, but no longer than 1 hr.
8. Read absorbance of each sample at 750 nm. The absorbances are stable for at least 1 hr.
9. Plot absorbance measurements as a function of concentration for the standards.
10. Interpolate the concentration of the protein samples from the plot and sample absorbance measurements.



CHAPTER 9

First-Dimension IEF with IPG Strips

Tips for IEF

- Master 2-D separation techniques using the ReadyPrep™ 2-D starter kit (catalog #163-2105) before using your own samples. The kit contains premixed reagents, a standard sample, and a detailed and optimized protocol, which allows you to become familiar with the 2-D workflow and techniques while validating the performance of your 2-D system
- When preparing solutions, use clean and dust-free vessels to avoid keratin contamination
- Use highly purified laboratory water (conductivity <2 µS)
- Use deionized urea prepared with a mixed-bed ion exchange resin to avoid protein carbamylation by cyanate, which forms in old urea
- Do not heat urea-containing buffers to >37°C to avoid protein carbamylation

IPG Strip Rehydration and Sample Loading

Prior to their use in IEF, IPG strips must be rehydrated (with or without sample) to their original thickness with rehydration solution (Table 9.1), which is often the 2-D sample solution (see Chapter 8).

Tips for Rehydration and Sample Loading

- Rehydrate IPG strips for 12 hr–overnight at 20°C (or room temperature)
- After rehydration in a rehydration/equilibration tray, rinse and blot the IPG gel strips to remove excess rehydration solution before transferring to the focusing tray; otherwise, urea may crystallize on the surface of the IPG strips
- Moisten electrode wicks with deionized water. They should be moist, not wet

Table 9.1. Rehydration volumes and sample loads. Protein load recommendations are intended as a starting point, and the optimum amount for the sample must be determined empirically. For narrow-range IPG strips, use more protein (proteins outside the range will not remain on the strip). For single-pH-unit IPG strips, use up to 4–5 times more protein to improve the detection of low-abundance proteins.

	IPG Strip Length, cm				
	7	**11**	**17**	**18**	**24**
Rehydration solution	125 µl	200 µl	300 µl	315 µl	450 µl
Protein load					
Coomassie (Brilliant) Blue	50–100 µg	100–200 µg	200–400 µg	200–400 µg	400–800 µg
Fluorescent stains	5–100 µg	20–200 µg	50–400 µg	50–400 µg	80–800 µg
Silver stains	5–20 µg	20–50 µg	50–80 µg	50–80 µg	80–150 µg
Mineral oil	4 ml	5 ml	7 ml	7 ml	9 ml

Performing IEF

IPG Strip Rehydration in Rehydration/Equilibration Trays Followed by IEF

The instructions in this chapter pertain to the use of the PROTEAN® i12™ cell and accessories. For more details about the components of this system and their assembly and use, please refer to the PROTEAN i12 cell instruction manual (bulletin 10022069).

Protocol

1. Pipet the rehydration solution (with or without sample, see Table 9.1 for volumes and protein loads) along the center of the channel(s) of the i12 rehydration/equilibration tray. Take care not to introduce air bubbles when expelling the solution.
2. Using forceps, remove the cover sheet from the IPG strip, then gently place the IPG strip gel-side down onto the solution in the channel. Move the IPG strip back and forth slightly to ensure that the solution is distributed along its length and that the strip is not sticking to the bottom of the tray. Take care to avoid trapping air bubbles beneath the IPG strip.
3. Overlay each IPG strip with mineral oil to prevent evaporation and precipitation of urea during rehydration (see Table 9.1 for recommended volumes). Apply the mineral oil to both ends of the channel and allow it to flow toward the middle.
4. Cover the tray and leave it on a level bench overnight (12–18 hr) for complete rehydration.
5. Transfer the rehydrated IPG strips to the focusing tray for IEF (see below).

IEF with Gel-Side Up

The following protocol is for IPG strips that have been rehydrated in the presence of sample (in-gel sample loading).

Protocol

1. Using forceps, remove the IPG strips from the rehydration tray, remove excess mineral oil, and place the rehydrated IPG strips gel-side up in the channels of the focusing tray. Position the positive (+) ends of the IPG strips against the positioning stops in each channel.
2. **Recommended:** Wet the gel-side up wicks (notched) with distilled or deionized water and blot off excess water. Use two wicks per IPG strip: place a wick at each end of each IPG strip.
3. Position the electrode assemblies in the focusing tray and press down on the green tabs to snap the electrode assemblies into place. Place the focusing tray with the rehydrated IPG strips on the Peltier platform and connect the electrodes to the instrument.
4. Overlay each IPG strip with mineral oil (see Table 9.1 for recommended volumes).
5. Select or program the protocol(s) and start the run.

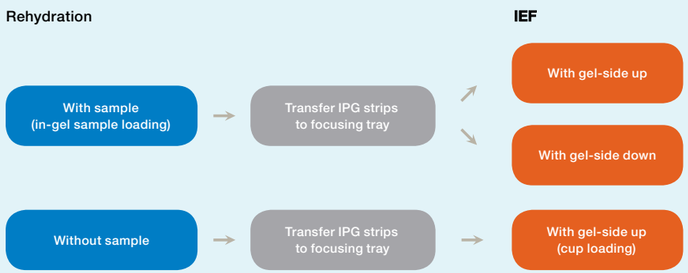


Fig. 9.1. Sample loading.

Performing IEF (contd.)

IEF with Gel-Side Down

The following protocol is for IPG strips that have been rehydrated in the presence of sample (in-gel sample loading).

Protocol

1. Position the electrode assemblies in the channels of the focusing tray and press down on the green tabs to snap the electrode assemblies into place.
2. **Recommended:** Wet the rectangular (gel-side down) wicks with distilled or deionized water and blot off excess water. Use two wicks per IPG strip: place a wick on top of each electrode.
3. Using forceps, place the rehydrated IPG strips gel-side down in the channels of the focusing tray. Position the positive (+) ends of the IPG strips against the positioning stops in each channel.
4. Place the focusing tray on the Peltier platform and connect the electrodes to the instrument.
5. Overlay each IPG strip with mineral oil (see Table 9.1 for recommended volumes).
6. Place the IPG strip retainers on top of the IPG strips at both the positive and the negative ends. Without IPG strip retainers in place, gases formed during electrolysis may lift IPG strips off the electrodes, interrupting electrical contact.
7. Select or program the protocol(s) and start the run.

Cup Loading (IEF with Gel-Side Up)

This protocol is for IPG strips that have been rehydrated in the absence of sample. Sample cups offer an alternative method of sample loading, and their use can often improve resolution, especially at extreme pH ranges. The PROTEAN i12 sample cup assembly consists of a sample cup holder that holds 1–12 disposable sample cups.

Protocol

1. Using forceps, place the rehydrated IPG strips gel-side up in the channels of the focusing tray. Position the positive (+) end of the IPG strips against the positioning stops in each channel.
2. **Recommended:** Wet the gel-side up electrode wicks (notched) with deionized water and blot off excess water. Use two wicks per IPG strip: place a wick at each end of each IPG strip.
3. Position the electrode assemblies in the focusing tray and press down on the green tabs to snap the electrode assemblies into place. Place the focusing tray with the rehydrated IPG strips on the Peltier platform, and connect the electrodes to the instrument.
4. Prepare the sample cup assembly by placing the sample cups into the slots of the sample cup holder corresponding to the channel with the rehydrated IPG strip.
5. Clamp the sample cup assembly onto the edges of the focusing tray, on top of the IPG strips and next to either electrode. Placement depends on the pH gradient and the sample. In general, focusing is most effective towards the end of the IPG strip opposite the site of the sample cup placement. Use anodic sample cup placement when using basic pH ranges or when resolution of basic proteins is desired.
6. Load 25–250 µl of sample into the sample cups (larger volumes of dilute samples of up to 400 µl may be loaded). Overlay both the sample in the sample cup and the IPG strip with mineral oil.
7. Select or program the protocol(s) and start the run.

IPG Strip Rehydration in the Focusing Tray Followed by IEF

For rehydration and IEF in the focusing tray, place the IPG strip gel-side down on top of the rehydration solution in the focusing tray. Rehydration can be programmed as a part of the IEF run, and the protocols can be programmed next. Alternatively, the strips can be rehydrated independently and the protocol(s) started when most convenient.

Protocol

1. Position the electrode assemblies in the focusing tray.
2. Pipet the rehydration solution containing the protein sample along the center of the channel(s) of the focusing tray (see Table 9.1 for recommended volumes and protein loads). Do not introduce air bubbles when expelling the solution.
3. Using forceps, remove the cover sheet from the IPG strip, then gently place the IPG strip gel-side down onto the sample in the channel of the tray. To ensure even rehydration, move the IPG strip back and forth slightly to distribute the solution along its length. Check that no bubbles are trapped beneath the strips and that some rehydration solution extends beyond the electrode contacts.
4. Place the focusing tray with the IPG strips on the Peltier platform and connect the electrodes to the instrument.
5. Immediately overlay each IPG strip with mineral oil to prevent evaporation and precipitation of urea during rehydration. Apply the mineral oil to both ends of the channel and allow it to flow toward the middle. See Table 9.1 for recommended volumes of mineral oil.
6. Position the IPG strip retainers on top of the IPG strips at both the anode and the cathode to maintain electrical contact with the IPG strips. Without the IPG strip retainers, gases formed during electrolysis may lift the IPG strips off the electrodes, interrupting electrical contact.
7. Rehydration in the focusing tray with in-gel sample application can be programmed as a part of the IEF run or be performed separately.

   To program rehydration as part of the run:

   a. Select or program the protocol(s) for the lanes containing IPG strips
   b. Program the global rehydration conditions. If electrode wicks are used, include a post-rehydration pause to insert electrode wicks when the rehydration step is completed
   c. Start the run

   For rehydration not programmed as part of the run, leave the tray on the Peltier platform or on a level bench overnight (11–16 hr) for complete rehydration.
8. Start the run (perform IEF).

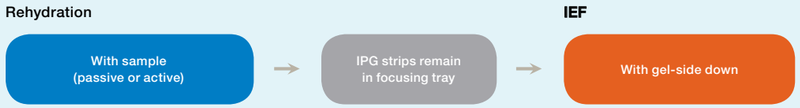


Fig. 9.2. Rehydration in the focusing tray.

IEF Programming Recommendations

The protocols and settings described are for IEF using the PROTEAN i12 IEF cell. Preprogrammed protocols serve as convenient starting points for optimization of IEF conditions (Tables 9.2 to 9.5).

- The recommended focusing temperature for most samples is 20°C
- For better sample entry, start IEF with a low voltage gradient (200 V for 30–180 min) and limit current to 50 µA per IPG strip for the whole run
- Focusing time depends on gel length, pH gradient, gel additives, and protein amount loaded. Vertical streaking is often caused by overfocusing — isoelectric precipitation (pI fallout) increases with focusing time. For this reason, do not conduct first-dimension IEF for any longer than is necessary
- After completion of the IEF run, IPG strips should be stored frozen at –80°C in rehydration trays or immediately applied to a second-dimension SDS-gel. Frozen IPG strips can be stored for about 3–6 months

Table 9.2. Preprogrammed protocols for 7 cm ReadyStrip™ IPG strips.

Protocol Name	Step	Voltage, V	Ramp	Time	Units
7 cm pH 3–10 R **7 cm pH 3–10 NL R** **7 cm pH 4–7 R** **7 cm pH 5–8 R**	1	4,000	Rapid	15,000	Vh
	2	500		Hold	
7 cm pH 3–10 G **7 cm pH 3–10 NL G** **7 cm pH 4–7 G** **7 cm pH 5–8 G**	1	250	Rapid	0:20	HH:MMr
	2	4,000	Gradual	1:00	HH:MM
	3	4,000	Rapid	15,000	Vh
	4	500		Hold	
7 cm pH 3–6 R	1	4,000	Rapid	10,000	Vh
	2	500		Hold	
7 cm pH 3–6 G	1	250	Rapid	0:15	HH:MMr
	2	4,000	Gradual	1:00	HH:MM
	3	4,000	Rapid	20,000	Vh
	4	500		Hold	
7 cm pH 3.9–5.1 **7 cm pH 4.7–5.9**	1	250	Rapid	0:15	HH:MMr
	2	4,000	Gradual	1:00	HH:MM
	3	4,000	Rapid	20,000	Vh
	4	500		Hold	
7 cm pH 5.5–6.7 **7 cm pH 6.3–8.3**	1	250	Rapid	0:15	HH:MMr
	2	4,000	Gradual	1:00	HH:MM
	3	4,000	Rapid	25,000	Vh
	4	500		Hold	
7 cm pH 7–10 R	1	4,000	Rapid	16,000	Vh
	2	500		Hold	
7 cm pH 7–10 G	1	250	Rapid	0:15	HH:MMr
	2	4,000	Gradual	1:00	HH:MM
	3	4,000	Rapid	16,000	Vh
	4	500		Hold	

R = rapid, G = gradual

Table 9.3. Preprogrammed protocols for 11 cm ReadyStrip IPG strips.

Protocol Name	Step	Voltage, V	Ramp	Time	Units
11 cm pH 3–10 R **11 cm pH 3–10 NL R** **11 cm pH 4–7 R** **11 cm pH 5–8 R**	1	8,000	Rapid	26,000	Vh
	2	750		Hold	
11 cm pH 3–10 G **11 cm pH 3–10 NL G** **11 cm pH 4–7 G** **11 cm pH 5–8 G**	1	250	Rapid	0:20	HH:MMr
	2	8,000	Gradual	1:00	HH:MM
	3	8,000	Rapid	26,000	Vh
	4	1,500		Hold	
11 cm pH 3–6 R	1	8,000	Rapid	20,000	Vh
	2	750		Hold	
11 cm pH 3–6 G	1	250	Rapid	0:20	HH:MMr
	2	8,000	Gradual	1:00	HH:MM
	3	8,000	Rapid	20,000	Vh
	4	750		Hold	
11 cm pH 3.9–5.1 **11 cm pH 4.7–5.9**	1	250	Rapid	0:20	HH:MMr
	2	8,000	Gradual	1:00	HH:MM
	3	8,000	Rapid	32,000	Vh
	4	750		Hold	
11 cm pH 5.5–6.7 **11 cm pH 6.3–8.3**	1	250	Rapid	0:20	HH:MMr
	2	8,000	Gradual	1:00	HH:MM
	3	8,000	Rapid	40,000	Vh
	4	750		Hold	
11 cm pH 7–10 R	1	8,000	Rapid	29,000	Vh
	2	750		Hold	
11 cm pH 7–10 G	1	250	Rapid	0:20	HH:MMr
	2	8,000	Gradual	1:00	HH:MM
	3	8,000	Rapid	29,000	Vh
	4	750		Hold	

R = rapid, G = gradual

IEF Programming Recommendations (contd.)

Table 9.4. Preprogrammed protocols for 17 and 18 cm ReadyStrip IPG strips.

Protocol Name		Step	Voltage, V	Ramp	Time	Units
17 cm pH 3–10 R **17 cm pH 3–10 NL R** **17 cm pH 4–7 R** **17 cm pH 5–8 R**	**18 cm pH 3–10 R** **18 cm pH 3–10 NL R** **18 cm pH 4–7 R** **18 cm pH5–8 R**	1	10,000	Rapid	43,000	Vh
		2	1,000		Hold	
17 cm pH 3–10 G **17 cm pH 3–10 NL G** **17 cm pH 4–7 G** **17 cm pH 5–8 G**	**18 cm pH 3–10 G** **18 cm pH 3–10 NL G** **18 cm pH 4–7 G** **18 cm pH 5–8 G**	1	250	Rapid	0:30	HH:MMr
		2	10,000	Gradual	2:00	HH:MM
		3	10,000	Rapid	43,000	Vh
		4	1,000		Hold	
17 cm pH 3–6 R	**18 cm pH 3–6 R**	1	10,000	Rapid	32,000	Vh
		2	1,000		Hold	
17 cm pH 3–6 G	**18 cm pH 3–6 G**	1	250	Rapid	0:30	HH:MMr
		2	10,000	Gradual	2:00	HH:MM
		3	10,000	Rapid	32,000	Vh
		4	1,000		Hold	
17 cm pH 3.9–5.1 **17 cm pH 4.7–5.9**	**18 cm pH 3.9–5.1** **18 cm pH 4.7–5.9**	1	250	Rapid	0:30	HH:MMr
		2	10,000	Gradual	2:00	HH:MM
		3	10,000	Rapid	50,000	Vh
		4	1,000		Hold	
17 cm pH 5.5–6.7 **17 cm pH 6.3–8.3**	**18 cm pH 5.5–6.7** **18 cm pH 6.3–8.3**	1	250	Rapid	0:30	HH:MMr
		2	10,000	Gradual	2:00	HH:MM
		3	10,000	Rapid	63,000	Vh
		4	1,000		Hold	
17 cm pH 7–10 R	**18 cm pH 7–10 R**	1	10,000	Rapid	46,000	Vh
		2	1,000		Hold	
17 cm pH 7–10 G	**18 cm pH 7–10 G**	1	250	Rapid	0:30	HH:MMr
		2	10,000	Gradual	2:00	HH:MM
		3	10,000	Rapid	46,000	Vh
		4	1,000		Hold	

R = rapid, G = gradual

Table 9.5. Preprogrammed protocols for 24 cm ReadyStrip IPG strips.

Protocol Name	Step	Voltage, V	Ramp	Time	Units
24 cm pH 3–10 R **24 cm pH 3–10 NL R** **24 cm pH 4–7 R** **24 cm pH 5–8 R**	1	10,000	Rapid	60,000	Vh
	2	1,500		Hold	
24 cm pH 3–10 G **24 cm pH 3–10 NL G** **24 cm pH 4–7 G** **24 cm pH 5–8 G**	1	250	Rapid	0:30	HH:MMr
	2	10,000	Gradual	2:00	HH:MM
	3	10,000	Rapid	60,000	Vh
	4	1,500		Hold	
24 cm pH 3–6 R	1	10,000	Rapid	44,000	Vh
	2	1,500		Hold	
24 cm pH 3–6 G	1	250	Rapid	0:30	HH:MMr
	2	10,000	Gradual	2:00	HH:MM
	3	10,000	Rapid	44,000	Vh
	4	1,500		Hold	
24 cm pH 3.9–5.1 **24 cm pH 4.7–5.9**	1	250	Rapid	0:30	HH:MMr
	2	10,000	Gradual	2:00	HH:MM
	3	10,000	Rapid	70,000	Vh
	4	1,500		Hold	
24 cm pH 5.5–6.7 **24 cm pH 6.3–8.3**	1	250	Rapid	0:30	HH:MMr
	2	10,000	Gradual	2:00	HH:MM
	3	10,000	Rapid	70,000	Vh
	4	1,500		Hold	
24 cm pH 7–10 R	1	10,000	Rapid	63,000	Vh
	2	1,500		Hold	
24 cm pH 7–10 G	1	250	Rapid	0:30	HH:MMr
	2	10,000	Gradual	2:00	HH:MM
	3	10,000	Rapid	63,000	Vh
	4	1,500		Hold	

R = rapid, G = gradual

CHAPTER 10

Second-Dimension SDS-PAGE

Tips for SDS-PAGE

- Ensure that gels have the same composition by either using precast gels, which are manufactured in lots and so are virtually identical, or hand casting the gels at the same time in a multi-casting chamber
- Save time by preparing the overlay solution and running buffers during the 10 min equilibration incubations
- Vertical streaking on second-dimension gels is often caused by gaps between the IPG strips and the gels. Ensure that the second-dimension gel has a straight and level top edge, and that the IPG strip is in direct contact with the gel along its entire length
- When preparing running buffers, make the solution as specified in the protocol and do not titrate to a pH. The ion balance is set by the concentration of reagents; adjusting the pH alters this balance and leads to undesirable results
- Do not reuse running buffers
- Use 5–10 V per cm of gel for 10 to 30 min during sample entry (until the sample has concentrated at the starting point of the separation gel). Then continue with the voltage setting recommended in the instruction manual for the electrophoresis system you are using
- Use the voltage setting recommended in the instruction manual for the electrophoresis system you are using; excessive voltage leads to decreased resolution and distortions
- When running multiple cells, use the same voltage for multiple cells as you would for one cell. Be aware that the current drawn from the power supply will double with two — compared to one — cells. Use a power supply that can accommodate this additive current and set the current limit high enough to permit this additive function
- To maximize reproducibility, maintain the temperature of the electrophoresis buffer at about 20°C with the help of a recirculating cooler

IPG Strip Equilibration

Equilibrate the IPG strips twice, each time for 10 min, in two different equilibration buffers. Use disposable rehydration/equilibration trays for this purpose.

Reagents

Tris-HCl buffer (25 ml)
1.5 M Tris-HCl (pH 8.8)

Dissolve 4.55 g of Tris base in ~20 ml of deionized or distilled H_2O. Adjust the pH of the solution with diluted HCl and adjust the volume to 25 ml with distilled or deionized H_2O.

Equilibration stock buffer (500 ml)
6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS in 0.05 M Tris-HCl buffer, (pH 8.8). Pre-prepared equilibration buffers can also be purchased.

Combine 180 g of urea, 150 g of glycerol, 10 g of SDS, and 16.7 ml of Tris-HCl buffer. Dissolve in deionized distilled H_2O and adjust the volume to 500 ml. Store frozen.

Equilibration buffer 1 (10 ml)
Add 100 mg of DTT to 10 ml of equilibration stock buffer.

Equilibration buffer 2 (10 ml)
Add 400 mg of iodoacetamide to 10 ml of equilibration stock buffer.

Protocol

1. Place one IPG strip gel-side up in each channel of a rehydration/equilibration tray, and fill the channels with the recommended volume of equilibration buffer.
2. Incubate with gentle agitation for 10 min, then decant.
3. Fill the channels with the recommended volume of equilibration buffer 2, and incubate again for 10 min.
4. After equilibration, remove the IPG strips and briefly rinse with the SDS-PAGE running buffer you will be using. This step rids the IPG strip of excess iodoacetamide and serves to lubricate the IPG strip for placement on the second dimension.

Table 10.1. Recommended equilibration volumes.

IPG Strip Length	7 cm	11 cm	17 cm	18 cm	24 cm
Equilibration buffer 1	2.5 ml	4 ml	6 ml	6 ml	8 ml
Equilibration buffer 2	2.5 ml	4 ml	6 ml	6 ml	8 ml

10 min is recommended for each equilibration step.

Sealing IPG Strips onto SDS-PAGE Gels

In this stage, the equilibrated IPG strips are placed on the top of polyacrylamide gels. This enables smooth movement of the focused proteins into the gel for separation by SDS-PAGE.

Reagents

Agarose solution (0.5% [w/v]): Suspend 0.5 g of low-melting agarose (low electroendosmosis, EEO) in 100 ml of SDS-PAGE running buffer, and dissolve it in a boiling water bath or in a microwave oven. Add a few crystals of bromophenol blue (or 100 µl of 1% bromophenol blue) to color the solution slightly.

The agarose solution can be aliquoted into sealed 1.5 ml or 2.0 ml plastic tubes, which can then be melted individually in a 100°C heat block when needed.

Caution: Wear protective gloves, goggles, and a lab coat when handling molten agarose. SDS in the molten agarose can cause the solution to bubble over. Molten agarose and the vessel containing it can cause severe burns if not handled carefully.

Molecular weight standards: SDS-PAGE standards can be applied to gels that have no reference lane. Trim a thin filter paper to ~4 × 5 mm and pipet 10 µl of SDS-PAGE standards onto the wick. Remove excess solution with filter paper. Alternatively, use Precision Plus Protein™ standard plugs, which can be used on vertical 2-D gels with or without a reference well.

Protocol

1. Position the second-dimension gel cassette so that it is leaning slightly backwards (approximately 30° from vertical). Use AnyGel™ stands, if available.
2. Place the equilibrated IPG strip (anodic side on the left) onto the long plate with the plastic backing against the plate.
3. Slide the strip between the plates using a spatula to push against the plastic backing. Ensure that the plastic backing remains fully in contact with the long plate and be careful not to damage the gel with the spatula. Make sure the IPG strip is positioned directly on top of the second-dimension gel without any bubbles in the interface between the two gel surfaces.
4. **Optional:** Slip a wick soaked with molecular weight standards or use a Precision Plus Protein standard plug in the slot in the gel sandwich next to or overlapping an end of the IPG strip.
5. To secure the strip in place, overlay it with molten agarose solution. Use warm molten agarose, as hot agarose may accelerate decomposition of the urea in the equilibration buffer. Avoid trapping air bubbles between the IPG strip and second-dimension gel. Dislodge any bubbles by tapping the plastic backing on top of the strip.
6. Stand the gel upright and allow the agarose to set for 5–10 min before loading the gel into the electrophoresis cell.

SDS-PAGE

Buffers and Solutions

This step requires the use of running buffer appropriate for the gel chemistry you are using.

SDS-PAGE Running Buffer (Tris-HCl and TGX™ formulations)

Prepare sufficient 1× Tris/glycine/SDS running buffer to run the number of gels in the system selected:

1 L of 1× Tris/glycine/SDS (25 mM Tris, 192 mM glycine, 0.1% SDS)

Tris base	3.03 g
Glycine	14.4 g
SDS	1.0 g
Distilled or deionized H_2O	to 1 L

Alternatively, dilute 10× stock solution (catalog #161-0732) to the desired volume.

Protocol

Perform SDS-PAGE according to the running conditions specified for the electrophoresis system you are using. In general:

1. Insert the gel cassettes in the electrophoresis apparatus and fill the buffer chamber(s) with SDS running buffer. SDS running buffer temperature should be kept constant at 20°C if the chamber design allows for external cooling.
2. Connect the electrophoresis cell to a power supply and perform electrophoresis at 5–10 V per cm of gel until the sample has concentrated at the starting point of the separation gel. Then continue with the voltage settings recommended by the instruction manual for the electrophoresis system you are using.
3. After electrophoresis, carefully open the cassettes and use a spatula to separate the agarose overlay, including the IPG strip, from the polyacrylamide gel.
4. Carefully peel the gel from the cassette and place it in a container with fixative or staining solution, depending on the staining procedure used (see Chapter 11).

CHAPTER 11

Protein Detection

Tips for Total Protein Staining

- Stain gels at room temperature with gentle agitation (for example, on an orbital shaker), making sure the gel is completely covered with stain solution at all times
- Use any convenient glass or plastic container that is appropriate to the method chosen. Use glass containers with silver staining methods or with Flamingo™ stain. Use plastic trays with SYPRO Ruby stain
- Use Bio-Rad's Dodeca™ stainers for high-throughput staining
- Wear gloves during the staining process, and handle gels only by the edges and corners. Wet gloves with water or buffer before handling the gel to keep the gel from sticking and tearing
- Use clean and dust-free containers for gel staining. Place a lid on the container to avoid contamination of the staining solution
- Use pure chemicals and highly purified water (conductivity <2 µS)
- When performing gel staining with fluorescent dyes, cover the staining tray with foil during incubations
- Fluorescent dyes like Flamingo and Oriole™ fluorescent gel stains have a higher dynamic range than Coomassie (Brilliant) Blue or silver staining techniques and are, therefore, recommended for quantitative protein analysis
- Gels stained with fluorescent dyes can be counterstained with Bio-Safe™ Coomassie stain for further reference and to enhance sensitivity of the Coomassie stain
- Silver staining is not generally recommended when protein spots will be identified by mass spectrometry, though some formulations are compatible with mass spectrometry at the expense of promised sensitivity. Use Bio-Safe Coomassie or fluorescent dyes like Flamingo or Oriole instead
- As an alternative to drying gels, seal them in zip-top plastic bags in either water or, for long-term storage, water with 0.005% sodium azide. Fill the bag with water, insert the gel, expel the water, and seal the bag

Long-Term Storage of Stained Gels

Gels stained with a visible stain can serve as a permanent record of the SDS-PAGE separation. Stained gels may be stored indefinitely when dried between cellophane sheets. To dry stained gels, the gel is placed on a sheet of wet cellophane. A second sheet of wet cellophane is carefully laid over the gel with care taken not to introduce bubbles or wrinkles. The gel, sandwiched between two sheets of wet cellophane, is clamped into a frame and allowed to dry.

The most common problem associated with drying gels is cracking. Cracking is best prevented by soaking the gel for at least 30 min in a 2% (w/v) solution of glycerol in water prior to drying. Alternatively, a commercially available gel-drying solution may be used.

Total Protein Staining

For more detailed instructions, refer to the respective instruction manuals.

Bio-Safe Coomassie Stain

Instruction manual: bulletin 4307051.

Protocol

1. Wash gels three times for 5 min each in distilled or deionized H_2O.
2. Remove water from staining container and add Bio-Safe Coomassie stain to completely cover the gel. Agitate for at least 1 hr.
3. Rinse in distilled or deionized H_2O for at least 30 min. Stained gels can be stored in water.

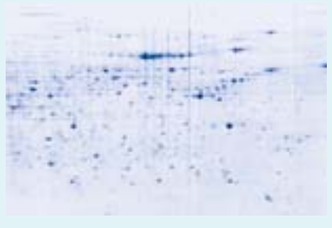


Fig. 11.1. 2-D gel stained with Bio-Safe Coomassie stain.

Flamingo Fluorescent Gel Stain

Instruction manual: bulletin 10003321. Refer to Table 11.1 for solution volumes.

Protocol

1. Place gel in a staining tray with fixing solution (40% ethanol, 10% acetic acid). Cover the tray and agitate gently for at least 2 hr.
2. Pour off the fixing solution and add 1× stain solution (dilute 1 part Flamingo fluorescent gel stain with 9 parts deionized or distilled H_2O). Cover the tray and agitate gently. Stain for at least 3 hr.
3. **Optional background reduction:** Carefully pour off the stain solution and replace with an equal volume of 0.1% (w/v) Tween 20. Cover the tray and agitate gently for 10 min.
4. Rinse gel with deionized or distilled H_2O prior to imaging.

Table 11.1. Flamingo fluorescent gel stain.

Gel size	Volume of fixing solution per gel	Volume of staining solution per gel
Mini (8.6 × 6.8 cm)	100 ml	50 ml
Midi (13.3 × 8.7 cm)	200 ml	100 ml
Large (16 × 16 cm or 16 × 20 cm)	500 ml	250 ml
Larger (25.6 × 23 cm)	1,000 ml	500 ml

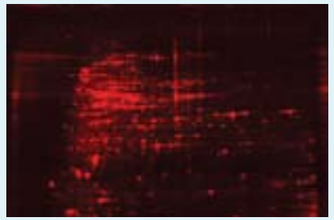


Fig. 11.2. 2-D gel stained with Flamingo stain.

Total Protein Staining (contd.)

Oriole Fluorescent Gel Stain

Instruction manual: bulletin 10017295. Refer to Table 11.2 for solution volumes.

Protocol

1. If using the 5 L configuration, prepare the Oriole stain solution by adding 400 ml of methanol to the 1 L bottle of diluent. Then add 10 ml of Oriole fluorescent gel stain concentrate and mix well by shaking.

Note: Do not fix or wash gel prior to staining. This will make staining less sensitive.

2. Place gel in a staining tray with Oriole fluorescent gel stain. Cover the tray and agitate for ~1.5 hr. For best results, do not leave gel in stain for more than 2 hr.

3. Rinse the gel in deionized distilled H_2O prior to imaging. Destaining is not necessary.

Table 11.2. Oriole fluorescent gel stain.

Gel size	Volume of staining solution per gel
Mini (8.6 × 6.8 cm)	50 ml
Midi (13.3 × 8.7 cm)	100 ml
Large (16 × 16 cm or 16 × 20 cm)	250 ml
Larger (25.6 × 23 cm)	500 ml

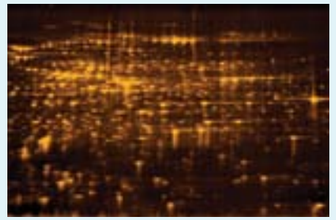


Fig. 11.3. 2-D gel stained with Oriole stain.

SYPRO Ruby Protein Gel Stain

Instruction manual: bulletin 4006173. Refer to Table 11.3 for solution volumes.

Protocol

1. Wash the gel in one of the following gel fixing solutions for 30 min:
   - 10% methanol, 7% acetic acid
   - 25% ethanol, 12.5% trichloroacetic acid
   - 10% ethanol, 7% acetic acid
   - 50% ethanol, 3% acetic acid
   - 40% ethanol, 10% acetic acid

2. Remove the wash solution and cover the gel with SYPRO Ruby protein gel stain. In general, use ~10 times the volume of the gel. Using too little stain will reduce sensitivity.

3. Stain the gel with continuous gentle agitation for at least 3 hr for best sensitivity. Specific staining can be seen in 30–90 min. For convenience, gels can be left in the stain solution overnight (16–18 hr).

4. Rinse the gel in 10% methanol (or ethanol), 7% acetic acid for 30–60 min to decrease background fluorescence. Rinse the gel in water before imaging.

Table 11.3. SYPRO Ruby fluorescent gel stain.

Gel size	Volume of staining solution per gel
8 × 108 cm	50 ml
16 × 20 cm	330 ml
20 × 20 cm	500 ml

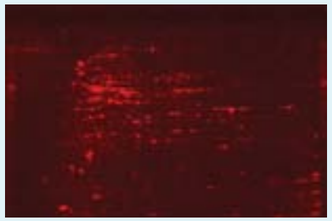


Fig. 11.4. 2-D gel stained with SYPRO Ruby stain.

Silver Stain Plus™ Kit

Instruction manual: bulletin LIT-442. Refer to Table 11.4 for solution volumes and incubation times.

Components:

- Fixative enhancer concentrate
- Silver complex solution
- Reduction moderator solution
- Image development reagent
- Development accelerator reagent
- Empty 1L bottle for development accelerator reagent

Protocol

1. Prepare the development accelerator reagent solution. Add the entire contents (50 g) of development accelerator reagent to deionized distilled H_2O and bring volume up to 1 L. Store at 4°C and use within 3 months.

2. **Fixative step.** Make fixative enhancer solution by mixing 50% (v/v) reagent-grade methanol, 10% (v/v) reagent-grade acetic acid, 10% (v/v) fixative enhancer concentrate, and 30% (v/v) deionized distilled H_2O. After gel electrophoresis, place gels in the fixative enhancer solution with gentle agitation.

3. **Water wash steps.** Decant the fixative enhancer solution from the staining vessel. Rinse gels in deionized or distilled H_2O with gentle agitation. Decant water and replace with fresh rinse water and rinse. Decant rinse water.

4. **Staining step.** To prepare staining solution, add 35 ml of deionized or distilled H_2O to a beaker or flask with a Teflon-coated stir bar. Add in the following order: 5.0 ml of silver complex solution, 5.0 ml of reduction moderator solution, and 5.0 ml of image development reagent. Immediately before use, quickly add 50 ml of development accelerator solution. Stir well. Stain gels with gentle agitation.

5. **Stop step.** After the desired staining is reached, place the gels in 5% acetic acid solution to stop the staining reaction. After stopping the reaction, rinse the gels in high purity water for 5 min. Then the gels are ready to be dried or photographed.

Table 11.4. Silver Stain Plus.

	Gel Thickness 0.75–1.0 mm		
Step	**Time**	**Mini Gel**	**Large Gel**
Fixative*	20 min	400 ml	800 ml
Water washes	10 min	400 ml	800 ml
Stain**	20 min	100 ml	300 ml
Stop	15 min	400 ml	400 ml

	Gel Thickness 1.5–3.0 mm		
Step	**Time**	**Mini Gel**	**Large Gel**
Fixative*	30 min	400 ml	800 ml
Water washes	20 min	400 ml	800 ml
Stain**	20 min	100 ml	300 ml
Stop	15 min	400 ml	400 ml

* Gels may be left in this solution indefinitely prior to staining; therefore, it is not necessary to carry out the entire procedure directly following electrophoresis.

** Stain until the desired intensity is reached. It may take at least 15 min before the first bands or spots become visible. Staining time is dependent on the sample and quantity loaded.

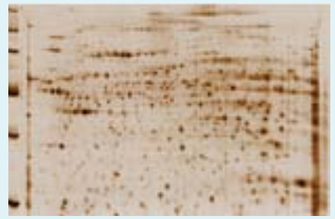
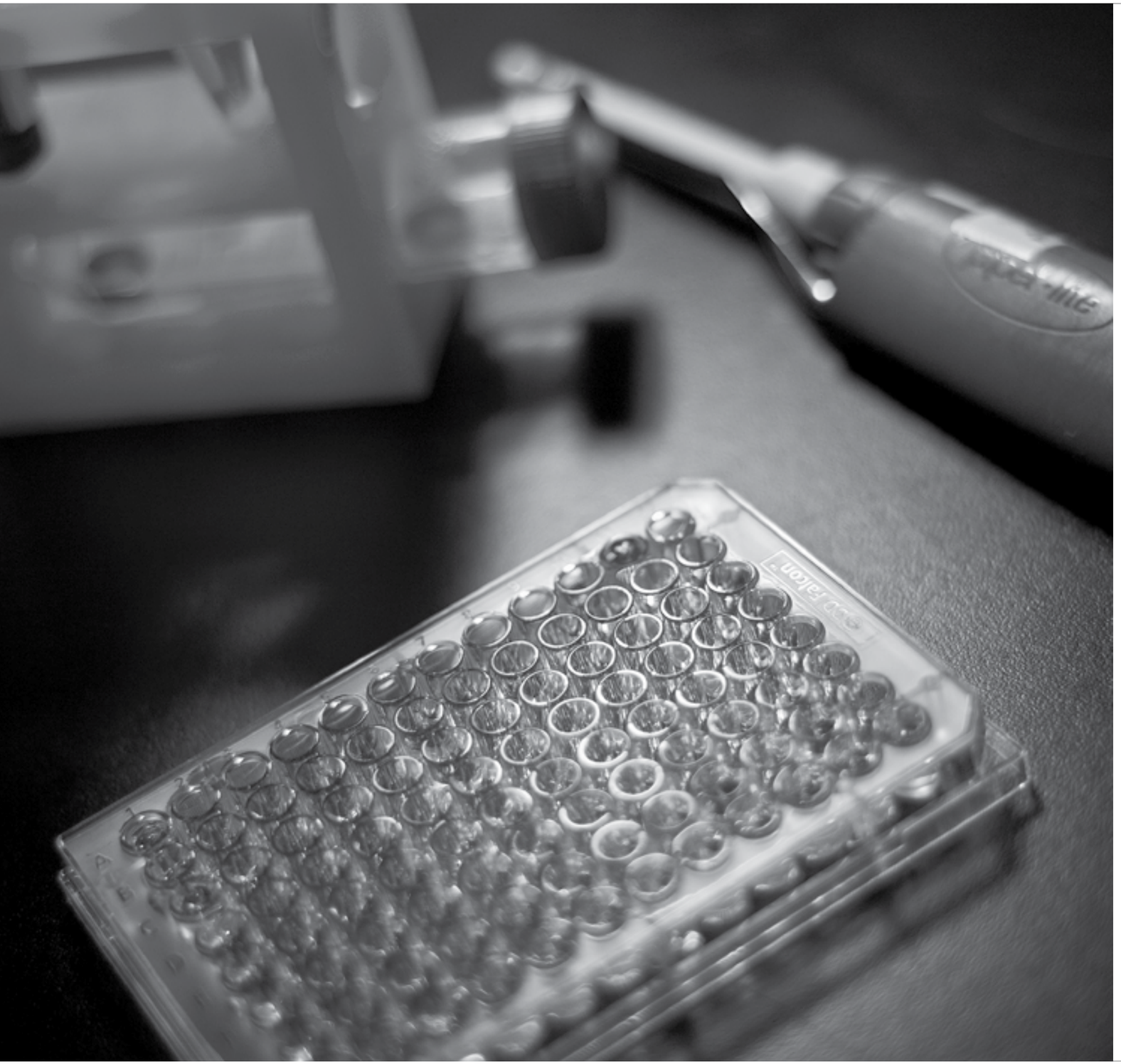


Fig. 11.5. 2-D gel stained with Silver stain.



CHAPTER 12

In-Gel Trypsin Digestion

Tryptic Digestion Protocol

This protocol for tryptic digestion of gel pieces (plugs) excised from SDS-PAGE gels is derived from the protocol described by Speicher et al. (2000). It can be used in conjunction with any of the non-silver stains described in this guide.

Reagents and Solutions

- Ammonium bicarbonate, NH_4HCO_3
- Acetonitrile
- Iodoacetamide
- HPLC-grade water (for example, Burdick and Jackson AH365)
- Trifluoracetic acid (TFA) (for example, Thermo Scientific 28904)
- Octyl β-D-glucopyranoside (for example, Sigma Aldrich 08001)
- Sequencing-grade modified trypsin, porcine (for example, Promega V5111)

Destaining buffer (50:50 ACN:0.2 M NH_4HCO_3)
Dissolve 158 mg of NH_4HCO_3 in 5 ml HPLC-grade water and add 5 ml acetonitrile.

50 mM NH_4HCO_3
Dissolve 79 mg of NH_4HCO_3 in 20 ml of water.

Reducing solution
Dissolve 555 mg of DTT in 3 ml of 50 mM NH_4HCO_3.

Alkylating solution
Dissolve 54 mg of iodoacetamide in 3 ml of 50 mM NH_4HCO_3.

Trypsin solution (20 µg/ml)
Dissolve 20 µg of trypsin in 1 ml of 50 mM NH_4HCO_3.

Extraction solvent
Combine 950 µl of 1% TFA with 50 µl of 1% octyl D-glucopyranoside.

Destaining Gel Plugs from Silver-Stained Gels (Pre-Treatment)

Gels that have been stained with a mass spectrometry–compatible silver stain benefit from an additional treatment to remove silver metal by oxidation.

All materials used should be ACS reagent grade or better.

Solution A (30 mM potassium ferricyanide)
To prepare 50 ml, dissolve 494 mg of potassium ferricyanide [$K_3Fe(CN)_6$] in 50 ml of water. This solution may be stored indefinitely at room temperature.

Solution B (100 mM sodium thiosulfate)
To prepare 50 ml, dissolve 791 mg of anhydrous sodium thiosulfate [$Na_2S_2O_3$] in 50 ml of water. This solution may be stored for one year in a sealed bottle.

Protocol
Prepare the silver destain solution just prior to use. It is good for only one use. Discard any excess.

1. Mix Solutions A and B in a 1:1 ratio. This is the silver destain solution.
2. Place each gel plug in a 0.5 ml or 1.5 ml plastic tube.
3. Add 50 µl of silver destain solution. Incubate 20 min at room temperature.
4. Using a laboratory pipet, remove the silver destain solution and add 50 µl of fresh solution.
5. Repeat steps 2 and 3 for a total of three treatments. Following the last incubation, remove the silver destain solution.
6. Proceed with the procedure described below.

General Destaining Protocol

1. Add 100 µl of destaining buffer to the gel plug and incubate for 30 min. Remove and discard the solution.
2. Repeat step 1 two more times.
3. Add 400 µl of destaining buffer to the gel plug and incubate overnight at room temperature.

Reduction and Alkylation Protocol

This step is not necessary for 2-D gel plugs if they have already been reduced and alkylated during the sample preparation or equilibration steps.

1. Remove destaining buffer and dehydrate the gel by adding 50 µl of acetonitrile. Incubate 10 min at room temperature and remove excess solution (for example, by aspiration).
2. Dry the gel piece for 30 min in a laminar flow hood.
3. Add 100 µl of reducing solution to the gel plug and incubate 30 min at room temperature. Remove excess liquid.
4. Add 100 µl of alkylating solution to the gel plug and incubate 30 min at room temperature in the dark. Remove excess liquid.

Digestion Protocol

1. Add 50 µl of acetonitrile to the gel plug and incubate for 10 min at room temperature. Remove excess liquid and proceed to digestion.
2. To the dried gel plug, add a volume of trypsin solution equivalent to the volume of the original hydrated plug (1.5 mm plug = 3.4 µl).
3. Incubate at room temperature for 10 min (center of gel will change from opaque to clear). If gel plugs aren't swollen, add a few more µl of trypsin solution and incubate for an additional 10 min.
4. Add enough 50 mM NH_4HCO_3 to cover the gel plug (~10 µl).
5. Incubate at 37°C for at least 3 hr.

Extraction Protocol

1. Remove trypsin solution from the gel plug, and store it in another vial.
2. To the gel plug, add 2–8 µl of extraction solvent. For MALDI-MS analysis, keep this volume as small as possible (2–3 µl). For LC-MS analysis, add 8 µl.
3. Incubate 30 min at room temperature.
4. Combine extraction solvent with trypsin solution.

PART III
Troubleshooting

Isoelectric Focusing

Problem	Cause	Solution
Initial low or zero current	Poor contact between IPG strips and electrodes	Make sure that the gel side of the IPG strip is in contact with the electrode
		For the gel-side down configuration with the PROTEAN® i12™ cell, use the IPG strip retainers
	Incomplete wetting of electrode wicks	Wet the electrode wicks with distilled or deionized H_2O until they are damp, but not soaking wet
	Incomplete IPG strip rehydration	Check the rehydration volumes and times for the lengths of IPG strips used
No current in any lane	No contact between the electrode assembly and IPG strips	Make sure that: ▪ The electrode assembly is properly seated in the focusing tray ▪ The IPG strips are positioned correctly, (for example, that the gel is in direct contact with the electrode)
	No contact between the electrode assembly and instrument	Make sure that: ▪ The gold contact pin of the negative (–) assembly is in direct contact with the cathode bar on the instrument ▪ The positive (+) assembly is completely inserted into the anode of the instrument
Voltage does not increase beyond initial low voltage steps	High levels of ionic contaminants in sample solution (optimum salt concentration is ~10 mM, though up to 40 mM can be tolerated)	Keep salt concentrations under 40 mM; if necessary, desalt the sample (for example with Micro Bio-Spin™ 6 columns or the ReadyPrep™ 2-D cleanup kit)
		Salt collects in electrode wicks, so replace electrode wicks from time to time (every 2 hr) during the initial low-voltage steps. Several hours may be needed for ionic contaminants to leave IPG strips
Voltage does not reach programmed value, or maximum voltage is reached very slowly. **Note: good focusing may be obtained even if programmed voltage is never reached**	Programmed voltage is too high for the pH range and length of IPG strip	Lower the voltage maximum set for the focusing step; the conductivity and the length and type of IPG strip determine the voltage maximum that can be reached
	Ampholyte concentration is too high. Up to 1% (v/v) Bio-Lyte® ampholytes may be used, but ampholytes increase conductivity; therefore, voltage will be lower with increasing concentrations	Lower the ampholyte concentration

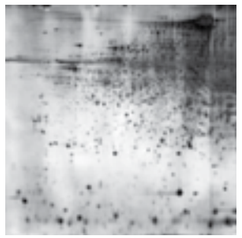
Isoelectric Focusing (contd.)

Problem	Cause	Solution
	Excess sample during rehydration did not enter gel, or IPG gels are overswelled with excess sample	Use correct rehydration volumes for the lengths of the IPG strips used
	Voltage is too high for the IPG strip size and pH gradient	Program Vh for the IEF step to ensure complete focusing of the sample
Large fluctuations in voltage and current	IPG strips contain poorly rehydrated regions, or IPG strips have dried out during the run	Check rehydration volumes and times
		Make sure that the rehydration solution is evenly distributed during rehydration and that the IPG strips are completely covered with mineral oil
Burning of strips	Current limit is too high	Use a current limit of 50 µA/IPG strip
	IPG strips have dried out	Make sure that the IPG strips are covered with mineral oil or equivalent
	Electrode wicks are too wet or contain incorrect electrode solution	Wet the electrode wicks with distilled or deionized H_2O until they are damp, not soaking wet
	Incorrect rehydration solution composition	Check the composition of the rehydration solution
Sample is leaking from the sample cups	Cup positioning is incorrect	When positioning the cup holder, make sure that it clicks into place at the edges of the focusing tray
	The cup is positioned in an area of the IPG strip that is not completely rehydrated	Make sure that the IPG strips are rehydrated evenly and thoroughly
	The cup is malfunctioning	Replace cup

SDS-PAGE

Problem	Cause	Solution
Low or zero current, and samples do not migrate into the gel	With a precast gel, the tape at the bottom of the gel cassette was not removed	Remove the tape
	Insufficient buffer in the inner or outer buffer chamber	Fill the inner and outer buffer chambers to ensure that the IPG well is completely covered
	Electrical disconnection	Check the electrodes and connections
Running time slower or faster than expected	Incorrect running buffer concentration or type	Check the buffer composition and type
Leaking from inner buffer chamber	Incomplete gasket seal	Wet the gasket with running buffer before use
	Improper assembly of the gel into the electrode/ companion assembly	▪ Ensure that the top edge of the short plate fits under the notch at the top of the gasket ▪ Ensure that the top of the short plate touches the green gasket

Total Protein Staining

Problem	Cause	Solution
Spots not visible (see 2-D Gel Evaluation, below)	No protein in the gel	Use another staining method to confirm that there is protein in the gel
	Malfunctioning imaging system or incorrect imaging parameters	Check the instrument manual for troubleshooting information, or contact the imaging instrument manufacturer
Poor staining load sensitivity	Insufficient protein in the gel	Repeat the experiment with a higher protein quantity
	Dirty staining trays (for example, with silver staining)	Clean the staining trays and other equipment thoroughly with laboratory glassware cleaner
	Insufficient stain volume	Follow the recommendations for stain volume appropriate to the gel size
	Insufficient staining time	Increase staining time
	Reuse of staining solution	To ensure quantitative reproducibility of a 2-D experiment, never reuse staining solution
High or uneven background staining	Dirty equipment or staining trays	Clean the staining trays and other equipment thoroughly with laboratory glassware cleaner
	Too much time in staining solution	Restrict the time in staining solution as recommended
		Wash the gel in water or respective destaining solution for >30 min
	Reagent impurities	Make sure that the water and reagents used for staining are of the highest possible quality
Diffuse, uneven background in silver-stained gel 	Insufficient washing	Perform more washing steps. Use purified laboratory water and clean staining trays
		Do not place too many gels in one tray. Fully immerse the gels in the staining solution; they should not stick to the staining tray
	Insufficient fixative (some uneven background stain is normal when using a silver stain. Due to migration of different chemicals and ions into the gels, some regions can be stained with different colors or intensities)	Apply a longer fixing procedure
	Contaminant(s) in the agarose overlay solution	Prepare fresh overlay solution

Total Protein Staining (contd.)

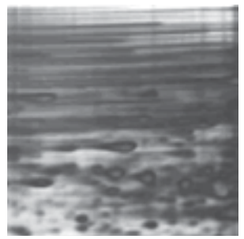
Problem	Cause	Solution
Speckles or blotches in the gel image	Particulate material from reagents, staining tray, dust, or gloves	Clean the staining trays and other equipment thoroughly with laboratory glassware cleaner
		Limit exposure of gels and staining solution to open air
		Use dust-free gloves, and handle gels only by the edges
Uneven staining	Insufficient shaking during staining	Agitate the gel during staining
Gel shrinkage	Some gel shrinkage occurs during staining	Transfer the gel to water

2-D Gel Evaluation*

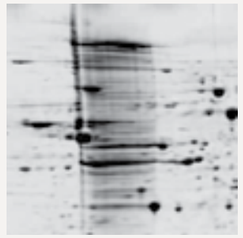
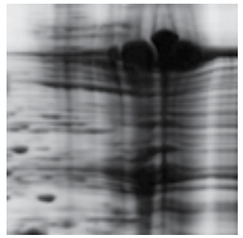
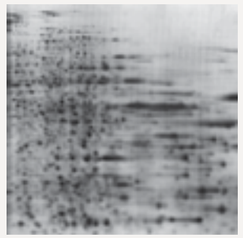
Problem	Cause	Solution
No Spots or Fewer Spots than Expected		
Across the gel	Insufficient sample was loaded	Check the sample concentration by protein assay
		Check that the protein assay is functioning properly and that it is not responding to interfering substances in your sample
	Insufficient sample entered the IPG strip	Start IEF at a low field strength
		Make sure that the IPG strips are in the correct orientation in the focusing tray
		Check that the orientation of electrical connections
		Increase the solubility strength of the 2-D sample solution; insoluble proteins will not enter the IPG strip
	Failure of detection reagents	Run a lane of unstained standards adjacent to the second-dimension separation. If the standards are not detected, check the expiration dates and the formulations of all detection reagents
	Staining method not sensitive enough	See Chapter 3 for sample loading recommendations dependent on the staining technique used
	Poor protein transfer from IPG strip to SDS-gel	Perform the first stage of SDS-PAGE at low voltage (50 V) for >20 min until the bromophenol blue front enters the separation gel (time depends on gel size)

* Also refer to Berkelman et al. (2004) and Bio-Rad Laboratories (2005).

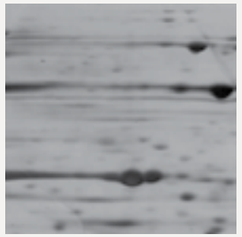
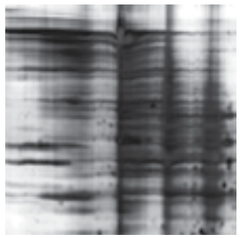
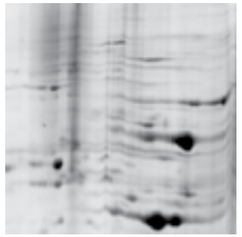
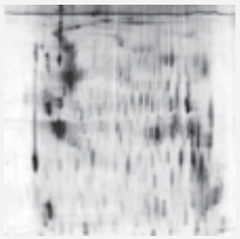
2-D Gel Evaluation (contd.)

Problem	Cause	Solution
No Spots or Fewer Spots than Expected		
In high molecular weight regions 	Sample may have undergone proteolysis prior to IEF	Include appropriate protease inhibitors and keep the sample on ice or in a cold room during sample preparation
	Insufficient equilibration	Incubate IPG strips in sufficient volumes of each equilibration buffer for up to 15 min with mild agitation
	Poor entry of high molecular weight proteins during rehydration (the pore size of the acrylamide in the IPG strip is very small during the early stages of rehydration)	Use active sample loading in the focusing tray or cup loading
	Poor entry of high molecular weight proteins into the second-dimension gel	Increase equilibration time (2 × 15 min)
Horizontal Streaking		
Across the entire gel 	Protein overloading	Use less sample
		Perform prefractionation to enrich the protein of interest and lower the amounts of other abundant proteins
		Use a longer IPG strip and larger gel size to allow for a greater protein load
	Proteins are not properly and stably solubilized	Solubilize proteins completely using a strong chaotropic extraction reagent. The concentrations of urea, thiourea, detergents, carrier ampholytes, and DTT are also critical. Every sample type typically requires a new sample preparation method
		Allow sufficient time for full denaturation and solubilization; for example, incubate the sample in the solubilization solution at room temperature for 1 hr before applying it to the IPG strip

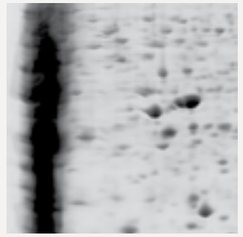
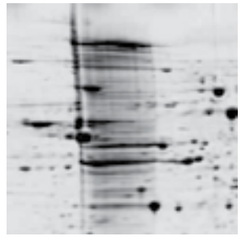
Horizontal Streaking (contd.)

Problem	Cause	Solution
Across the entire gel	DNA contamination	Treat the sample with a nuclease
		Make sure that the nuclease is active and that digestion is adequate; a very viscous sample implies that nuclease treatment has failed
	Incomplete focusing or overfocusing	Optimize the sample focusing time by running a time course. For example, run the sample on 6 IPG strips and remove an IPG strip at each time point (20 kV-hr, 30 kV-hr, 40 kV-hr, etc.)
	Incomplete IPG strip rehydration	Check the rehydration volumes and times for the lengths of IPG strips used
Partial 	Incomplete IPG strip rehydration	Check the rehydration volumes and times for the lengths of IPG strips used
		If the sample appears unevenly distributed, or if areas of the IPG strip are not wetted with sample, slide the IPG strip back and forth several times along the length of the channel in the focusing tray
Regional 	Protein overloading	Use less sample
		Perform prefractionation to enrich the protein of interest and lower the relative amounts of other abundant proteins
		Use a longer IPG strip and larger gel size to allow for a greater protein load
In the basic range of the gel 	Depletion of DTT in the basic range of the IPG strip	Treat the sample with the ReadyPrep reduction-alkylation kit prior to IEF

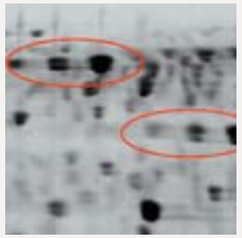
Horizontal Streaking (contd.)

Problem	Cause	Solution
Spots 	Incomplete IEF	Optimize the sample focusing time by running a time course. For example, run the sample on 6 IPG strips and remove an IPG strip at each time point (20 kV-hr, 30 kV-hr, 40 kV-hr, etc.)
Intermittent  	Contaminants such as salts, ionic detergents (for example, SDS), peptides, nucleic acids, lipids, polysaccharides, phenolic compounds	Use appropriate contaminant removal techniques, such as treatment with the ReadyPrep 2-D cleanup kit
Vertical Streaking		
Across the entire gel 	Leaking of the upper buffer reservoir (cathode) of the vertical electrophoresis unit	Prior to inserting the gel(s) into the vertical electrophoresis cell, wet the gaskets of the electrophoresis chamber with water or use a small amount of vacuum grease
	Incomplete equilibration	Increase equilibration time to 15 min
	Old DTT and iodoacetamide preparations used in equilibration	Use fresh reagents for the equilibration step

Vertical Streaking (contd.)

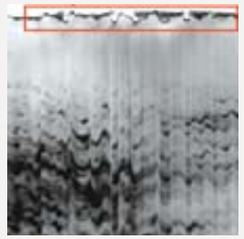
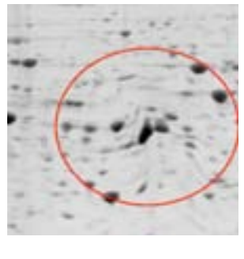
Problem	Cause	Solution
At one end of the gel (cup loading) 	Protein aggregation or precipitation caused by too much protein or sample loading problems	Dilute the sample to 3–5 µg/µl for cup loading
		Perform a protein assay prior to IEF to ensure correct protein load. The total amount of protein that should be loaded onto an IPG strip depends on the length of the strip and the stain that will be used to visualize the results
		Load the sample using in-gel sample loading
		Prolong the time on the initial low-voltage steps and increase the voltage gradually
	Field strength used for sample loading is too high	Reduce the field strength to ~10 V/cm IPG strip length
	Poor protein solubility	Increase the solubilizing strength of 2-D sample solution
Isolated streaking 	Improper rehydration of IPG strip	Check the rehydration volumes and times for the lengths of IPG strips used
		If the sample appears unevenly distributed, or if areas of the IPG strip are not wetted with sample, slide the IPG strip back and forth several times along the length of the channel in the focusing tray
Point streaking (handcast gels)	Dust or other particles in the gel solutions	Filter gel solutions through a 0.45 µm membrane and into a dust-free container
Vertical streaks connected to a spot	Insufficient binding of SDS to protein	Check the SDS concentration (>1%) in the equilibration solution
		Increase equilibration time: equilibrate IPG strips for 2 × 15 min
	Incorrect pH in resolving gel buffer; incorrect pH decreases mobility of protein-SDS complexes and causes vertical streaks	Ensure that the pH of the Tris buffer used for gel casting is 8.8
	Buffer leakage	Ensure that the upper buffer reservoir is not leaking

Vertical Streaking (contd.)

Problem	Cause	Solution
Twin vertical spots or vertical doublets 	Improper placement of the IPG strip onto the gel	Make sure that the focused IPG strip is in full contact with the gel
	Temperature gradient in the gel	Lower the power settings for the second-dimension SDS-PAGE run, especially when using cells that provide only one-sided cooling of the gel
		Use a better circulation system to improve heat dissipation during a run
Blank vertical stripes	Air bubble trapped in the agarose that joins the IPG strip to the top of the gel	Ensure that the 2-D gel has a straight, level top edge and that the IPG strip is in direct contact with the 2-D gel along its entire length. Squeeze out air bubbles by pressing on the plastic backing of the IPG strip
		Use a 0.5% agarose overlay solution to prevent the IPG strip from coming loose or moving. To minimize the number of bubbles in the overlay, melt the agarose overlay solution completely prior to loading
	Insufficient rehydration of a region of the IPG strip, or tears resulting from improper handling, resulting in the absence of focused protein in that region	Make sure that the IPG strip is not sticking to the bottom of the rehydration tray
		Check the integrity of rehydrated IPG strips prior IEF
	Focusing of an amphoteric nonprotein contaminant (for example, phospholipid or HEPES) prevents protein focusing around the pI of the contaminant	Apply sample cleanup

Vertical Streaking (contd.)

Problem	Cause	Solution
Blank stripes near pH 7	Excessive DTT (>50 mM) in the IPG sample solution	Lower the amount of DTT in the rehydration solution
Blank stripes at the electrodes, especially at the cathode	Salt buildup	Remove ionic contaminants from the samples with Bio-Rad´s ReadyPrep 2-D cleanup kit or by desalting
Blank vertical regions 	Interfering substances; impurities in the rehydration/sample solution	Remove contaminants from the samples with the ReadyPrep 2-D cleanup kit or by desalting
		Use high-quality reagents and chemicals for electrophoresis to minimize the risk of impurities. Replace chemicals of questionable or unknown shelf life, origin, or quality, as these products can also contribute to poor 2-D results
	Air bubble trapped in the agarose that joins the IPG strip to the top of the gel	Ensure that the 2-D gel has a straight, level top edge and that the IPG strip is in direct contact with the 2-D gel along its entire length. Squeeze out air bubbles by pressing on the plastic backing of the IPG strip
		Use a 0.5% agarose overlay solution to prevent the IPG strip from coming loose or moving. To minimize the number of bubbles in the overlay, melt the agarose overlay solution completely prior to loading

Problem	Cause	Solution
Other Problems		
Wavy spots 	Insufficient overlay solution used in gel casting	Overlay the gel with water-saturated butanol (n-butanol, l-butanol, or t-butanol) or t-amyl alcohol immediately after gel casting. These ensure that the gel has a clean, straight top edge
		Use precast gels
		Use the overlay recommended by the manufacturer of the electrophoresis cell
Localized wavy disturbance of spots 	Problems with casting second-dimension acrylamide gel: not evenly polymerized, gel cassette leaking, etc.	Optimize the APS and TEMED concentrations
		Degas solutions prior to the addition of APS/TEMED
		Perform casting at room temperature, warming the glass plates if necessary. Be aware that the polymerization process is temperature dependent. If the temperature is too low, polymerization may be compromised
		Use precast gels
Known proteins appearing as multiple spots or at the wrong position	Protein carbamylation	Do not prepare samples too far ahead of time in urea
		Do not expose urea-containing samples to high pH or temperatures that exceed 30°C
	Protein oxidation	Increase DTT concentration
	Protein proteolysis (during sample preparation)	Add protease inhibitors, perform manipulations as quickly as possible, and keep solutions as cold as possible

For further help or advice, please contact the Bio-Rad Technical Support department. In the United States, the Technical Support department is open Monday–Friday, 5:00 AM–5:00 PM, Pacific time.

Phone: 1-800-424-6723

Fax: 1-510-741-5802

Email: LSG_TechServ_US@bio-rad.com (for U.S. and international customers)

Online technical support and worldwide contact information are available at **www.consult.bio-rad.com**.

PART IV
Appendices

Appendix A

Glossary

%C Cross-linker concentration; weight percentage of cross-linker in a polyacrylamide gel. Effective pore size of a gel is a biphasic function of %C

%T Monomer concentration (acrylamide + cross-linker) in a gel (in g/100 ml). Effective pore size of a gel is an inverse function of %T, and gels can be made with a single, continuous %T throughout the gel (single-percentage gels), or they can be cast with a gradient of %T through the gel (gradient gels)

2-D electrophoresis Two-dimensional electrophoresis. Proteins are separated first according to isoelectric point (pI) by isoelectric focusing (IEF) and then according to size by SDS-PAGE, yielding a two-dimensional protein map of spots

2-Mercaptoethanol Reducing agent used for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding and to maintain all proteins in a fully reduced state. Also known as β-mercaptoethanol or BME

Acrylamide Monomer used with a cross-linker to form the matrix used for separating proteins or small DNA molecules

Ammonium persulfate (APS) Initiator used with TEMED (catalyst) to initiate the polymerization of acrylamide and bisacrylamide in making a polyacrylamide gel; $(NH_4)_2S_2O_8$

Ampholyte Amphoteric molecule that exists mostly as a zwitterion in a certain pH range. Ampholytes are used to establish a stable pH gradient for use in isoelectric focusing

Amphoteric Containing both acidic and basic groups

Anode Positively charged electrode. Negatively charged molecules (anions) move towards the anode, which is usually indicated by the color red

Anionic dye Negatively charged compound used as a stain; used in blotting to stain proteins immobilized on membranes such as nitrocellulose or PVDF

Antibody Immunoglobulin (Ig); protein produced in response to an antigen, which specifically binds the portion of the antigen that initiated its production

Assay Analysis of the quantity or characteristics of a substance

Background Nonspecific signal or noise that can interfere with the interpretation of valid signals

Bio-Spin® columns Family of Bio-Rad sample preparation products that includes the Bio-Spin® 6 and Micro Bio-Spin™ 6 columns; used for buffer exchange and desalting applications

Bis or bis-acrylamide A common cross-linker used with acrylamide to form a support matrix; N,N'-methylene-bis-acrylamide

Blot Immobilization of proteins or other molecules onto a membrane, or a membrane that has the molecules adsorbed onto its surface

Bromophenol blue Common tracking dye used to monitor the progress of electrophoresis

Carrier ampholytes Heterogeneous mixture of small (300–1,000 Da) polyamino-polycarboxylate buffering compounds that have closely spaced pI values and high conductivity. Within an electric field, they align according to pI to establish the pH gradient

Cathode Negatively charged electrode. Positively charged molecules (cations) move toward the cathode, which is usually indicated by the color black

Chaotropic agent Chemical that disrupts inter- and intramolecular interactions (for example, urea and thiourea)

CHAPS Zwitterionic detergent (having both positively and negatively charged groups with a net charge of zero) that is widely used for protein solubilization for IEF and 2-D electrophoresis; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

Comb Object used to cast wells in an agarose or acrylamide gel. In PAGE applications, square-bottom combs are inserted into the gel sandwich before polymerization to form square-bottomed wells

Coomassie (Brilliant) Blue Anionic dye used in the total protein staining of gels and blots and that comes in two forms: Coomassie (Brilliant) Blue G-250 differs from Coomassie (Brilliant) Blue R-250 by the addition of two methyl groups

Criterion™ cells, blotters, and gels Family of Bio-Rad products used for midi-format vertical electrophoresis; includes the Criterion and Criterion™ Dodeca™ cells, Criterion blotter, and Criterion precast gels

Cross-linker Molecule (for example, bis-acrylamide) used to link polymerizing monomer molecules together to form a netlike structure within the gel. The holes in the nets are called the pores, and the pore size is determined in part by the cross-linker concentration. The pores may or may not sieve the macromolecules

Cup loading Application of protein sample onto IPG strips through sample cups applied to the strips; can improve resolution at extremes of a pH gradient and improve uptake of basic proteins

***DC*™ assay kit** Bio-Rad's detergent-compatible protein assay kit

Depletion Reduction in the amount of high-abundance proteins relative to low-abundance proteins

Discontinuous buffer system Electrophoresis gel system that uses different buffers and sometimes different buffer compositions to focus and separate components of a sample. Discontinuous systems typically focus the proteins into tighter bands than continuous gel systems, allowing larger protein loads

Disulfide bond Chemical bond joining two sulfur atoms; commonly found in proteins, contributing to their secondary and tertiary structures

Dithiotheithol (DTT) Reducing agent used for cleavage of intra- and intermolecular disulfide bonds to achieve complete protein unfolding and to maintain all proteins in a fully reduced state

Electrophoresis Movement of charged molecules in a uniform electric field

Equilibration Preparation of protein separated in an IPG strip for second-dimension SDS-PAGE; reduces and alkylates sulfhydryl groups and saturates proteins with SDS

EXQuest™ spot cutter Bio-Rad's brand of spot cutter

Fractionation Separation of a sample into discrete parts for separate analysis; may improve detection of low-abundance proteins and reduce sample complexity

Glycine Amino acid used as the trailing or slow ion in SDS-PAGE according to Laemmli (Laemmli, 1970)

Gradient gel Gel with gradually changing monomer concentration (%T) in the direction of migration. In SDS-PAGE, gradients are used to separate wider molecular weight ranges of molecules than can be separated with single-percentage gels

Immobilized pH gradient (IPG) strips Strips in which buffering groups are covalently bound to an acrylamide gel matrix, resulting in stable pH gradients. This eliminates problems of gradient instability and poor sample loading capacity associated with carrier ampholyte–generated pH gradients

Immunoblotting Blot detection by antibody binding

Immunodetection Detection of a molecule by its binding to an antibody

In-gel sample application (In-gel rehydration) Sample application to the IPG strip during IPG strip rehydration; may be passive or active (in the presence of a low applied voltage)

Ionic strength Measure of the ionic concentration of a solution that affects its resistance

Isoelectric focusing (IEF) Electrophoresis technique that separates proteins according to their isoelectric point (pI)

Isoelectric point (pI) pH value at which a molecule carries no net electrical charge, or at which the negative and positive charges are equal

Leading ion Ion in a discontinous buffer system with a greater mobility, typically Cl^- (chloride ion)

MicroRotofor™ cells and kits Family of Bio-Rad sample preparation products, including the MicroRotofor liquid-phase IEF cell and MicroRotofor cell lysis kits

Monomer Unit that makes up a polymer (acrylamide is a monomer that is polymerized into polyacrylamide)

Mini-PROTEAN® cells and gels Family of Bio-Rad products used for mini-format vertical electrophoresis; includes the Mini-PROTEAN Tetra and Mini-PROTEAN® 3 Dodeca™ cells and Mini-PROTEAN precast gels

Molecular weight markers Mixtures of well-characterized or recombinant proteins used to help monitor separation as well as estimate the size of the proteins separated in a gel

Ohm's Law Describes the mutual dependence of three electrical parameters (V, voltage; I, current; R, resistance): $V = I \times R$

PAGE Polyacrylamide gel electrophoresis, a common method of separating proteins based on molecular weight

PDQuest™ software Bio-Rad's 2-D gel analysis software

Polyacrylamide Anticonvective, sieving matrix used in gel electrophoresis. Polyacylamide gels are cast using mixtures of acrylamide monomers with a cross-linking reagent, usually N,N'-methylenebisacrylamide (bis), both dissolved in buffer

Polyacrylamide gel electrophoresis (PAGE) Electrophoresis technique that uses polyacrylamide as the separation medium

PowerPac™ power supplies Family of Bio-Rad power supplies

Power supply Instrument that provides the electric power to drive electrophoresis and electrophoretic blotting experiments

Precision Plus Protein™ standards Bio-Rad's family of recombinant molecular weight markers

PROTEAN® cells Family of Bio-Rad products used for large-format vertical electrophoresis and isoelectric focusing; includes PROTEAN II xi, PROTEAN II XL, PROTEAN® Plus Dodeca™ cells, and the PROTEAN® i12™ IEF cell

ProteoMiner™ beads, reagents, and kits Protein enrichment technology that operates on the principle of dynamic range reduction; uses a bead-based library of combinatorial peptide ligands to enrich the amounts of medium- and low-abundance proteins relative to high-abundance proteins

Prestained standards Mixture of molecular weight marker proteins that have covalently attached dye molecules, which render the bands visible during electrophoresis and transfer

***RC DC*™ assay kit** Bio-Rad's reductant- and detergent-compatible protein assay kit

ReadyStrip™ IPG strips Bio-Rad's brand of IPG strips

ReadyPrep™ kits Bio-Rad's brand of sample preparation and 2-D electrophoresis kits and reagents

R_f value Relative distance a protein has traveled compared to the distance traveled by the ion front. The R_f value is used to compare proteins in different lanes and even in different gels. It can be used with standards to generate standard curves, from which the molecular weight or pI of an unknown may be estimated

Running buffer Buffer that provides the ions for the electrical current in an electrophoresis run. It may also contain denaturing agents. The running buffer provides the trailing ions in discontinuous electrophoresis

Sample solution Solution in which a sample is prepared or suspended prior to loading onto an IPG strip

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Separation of molecules by molecular weight in a polyacrylamide gel matrix in the presence of a denaturing detergent, such as sodium dodecyl sulfate (SDS). SDS denatures polypeptides and binds to proteins at a constant charge-to-mass-ratio. In a sieving polyacrylamide gel, the rate at which the resulting SDS-coated proteins migrate in the gel is relative only to their size and not their charge or shape

Sodium dodecyl sulfate (SDS) Anionic detergent that denatures proteins and binds to polypeptides in a constant weight ratio of 1.4 g/g of polypeptide (SDS:polypeptide)

Stain-free technology Protein detection technology involving UV-induced additive that modifies protein tryptophan residues. Continued exposure to UV light causes fluorescence of the modified proteins, which are then detected by a CCD imager. Sensitivity of this technique is generally equal to or better than Coomassie staining

Stained standards Mixture of molecular weight marker proteins that have covalently attached dye molecules; the bands are visible during electrophoresis and transfer

Standard Collection of molecules with known properties, such as molecular weight, isoelectric point, or concentration. Often used to create standard curves, from which the properties of an unknown may be determined

TGX™ Bio-Rad's Tris-glycine extended shelf life precast gels

Total protein stain Reagent that binds nonspecifically to proteins; used to detect the entire protein pattern on a blot or gel

Trailing ion Ion in a discontinous buffer system with a lower mobility, typically glycinate

Tris Organic component of buffer solutions that has an effective buffering range of pH 7.0–9.2; tris(hydroxymethyl)aminomethane

Triton X-100 Nonionic detergent widely used for protein solubilization (for IEF and 2-D electrophoresis)

Tween 20 Nonionic detergent; used in blot detection procedures as a blocking reagent or added to wash buffers to minimize nonspecific binding and background

Unstained standards Mixture of molecular weight marker proteins that do not have covalently attached dye molecules; the bands are invisible during electrophoresis and transfer, but are useful for molecular weight determination in stained gels

Urea Chaotrope usually included at rather high concentrations (9.5 M) in sample solubilization buffers for denaturing IEF and 2-D PAGE

Volt-hour (Vh) Voltage multiplied by time is used as a unit for the duration of an IEF run

Western blotting Immobilization of proteins onto a membrane and subsequent detection by protein-specific binding and detection reagents

Zwitterion Neutral molecule with positive and negative charges at different locations

Appendix B

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Bulletin 2895 Protein Blotting Guide

Bulletin 2998 Protein Standards Application Guide

Bulletin 3103 Removal of Abundant Myofilament Proteins from Rabbit Myocardium Using the ReadyPrep Protein Extraction Kit (Membrane I)

Bulletin 3110 Tips to Prevent Streaking on 2-D Gels

Bulletin 3131 The Rotofor System As a Prefractionation Device Used Prior to Electrophoresis

Bulletin 3133 Molecular Weight Determination by SDS-PAGE

Bulletin 3144 Using Precision Plus Protein Standards to Determine Molecular Weight

Bulletin 3145 Strategies for Protein Sample Preparation

Bulletin 5241 Important Factors Influencing Protein Solubility for 2-D Electrophoresis

Bulletin 5344 Fractionation by Liquid-Phase Isoelectric Focusing in the MicroRotofor Cell: Improved Detection of Low-Abundance Proteins

Bulletin 5398 Enriching Basic and Acidic Rodent Brain Proteins with Ion Exchange Spin Columns for Two-Dimensional Gel Electrophoresis

Bulletin 5754 Comparison of SYPRO Ruby and Flamingo Fluorescent Gel Stains with Respect to Compatibility with Mass Spectrometry

Bulletin 5782 In-Gel Protein Quantitation Using the Criterion Stain Free Gel Imaging System

Bulletin 5841 Quantitation of Serum and Plasma Proteins after Enrichment of Low-Abundance Proteins with the ProteoMiner Protein Enrichment System

Bulletin 5911 Mini-PROTEAN TGX Precast Gel: A Versatile and Robust Laemmli-Like Precast Gel for SDS-PAGE

Bulletin 5939 Overcoming the Coomassie Blues

Bulletin 6040 A Guide to Polyacrylamide Gel Electrophoresis and Detection

Bulletin 6138 PROTEAN i12 IEF System: Independent Voltage and Current Control Enables Optimization of First-Dimension IEF Conditions

Bulletin 6139 Versatile Separation Capabilities of the PROTEAN i12 IEF System

Bulletin 6140 Use of the PROTEAN i12 IEF System for In-Gel Peptide Fractionation Prior to LC-MS and Comparison with Off-Gel Fractionation

Bulletin 4006173 Instruction Manual, SYPRO Ruby Protein Stains

Bulletin 4307051 Instruction Manual, Bio-Safe Coomassie Stain

Bulletin 10003321 Instruction Manual, Flamingo Fluorescent Gel Stain

Bulletin 10017295 Instruction Manual, Oriole Fluorescent Gel Stain

Bulletin 10022069 Instruction Manual, PROTEAN i12 IEF System

Bulletin LIT-442 Instruction Manual, Silver-Stain Plus.

Appendix C

Ordering Information

Catalog #	Description
Protein Sample Preparation Kits and Reagents	
Protein Extraction	
163-2141	**MicroRotofor™ Cell Lysis Kit (Mammal)**, 15 preps, includes 50 ml protein solubilization buffer (PSB), ReadyPrep™ mini grinders (2 packs of 10 each)
163-2142	**MicroRotofor Cell Lysis Kit (Plant)**, 10 preps, includes 50 ml protein solubilization buffer (PSB), ReadyPrep 2-D cleanup kit (50 reaction size)
163-2143	**MicroRotofor Cell Lysis Kit (Yeast)**, 15 preps, includes 50 ml protein solubilization buffer (PSB), 15 ml yeast suspension buffer, 2 × 0.5 ml lyticase (1.5 U/µl)
163-2144	**MicroRotofor Cell Lysis Kit (Bacteria)**, 15 preps, includes 50 ml protein solubilization buffer (PSB), 25 ml bacteria suspension buffer, 1 ml lysozyme (1,500 U/µl)
163-2086	**ReadyPrep™ Protein Extraction Kit (Total Protein)**, 20 preps, general purpose protein preparation kit, includes strong detergent ASB-14
163-2146	**ReadyPrep Mini Grinders**, pkg of 20, 1.5 ml grinding tube, contains grinding resin and matching pestle, sufficient for twenty 100 mg extractions
163-2083	**ReadyPrep 2-D Rehydration/Sample Buffer 1**, 10 ml, protein solubilization reagent, includes 7 M urea, 2 M thiourea, 1% ASB-14, 40 mM Tris, 0.001% bromophenol blue
163-2106	**ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer**, 10 ml, protein sample buffer, includes 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® ampholyte, 0.001% bromophenol blue
163-2145	**Protein Solubilization Buffer (PSB)**, pkg of 1, strongly chaotropic protein solubilization buffer, contains NDSB 201, urea, thiourea, and CHAPS, makes 50 ml solution
163-2091	**ReadyPrep Proteomics Grade Water**, 500 ml
161-0730	**Urea**, 250 g
161-0719	**Tris**, 1 kg
161-0460	**CHAPS**, 1g
161-0611	**Dithiothreitol (DTT)**, 5 g
163-2101	**Tributylphosphine (TBP)**, 0.6 ml, 200 mM
163-2109	**Iodoacetamide**, 30 g
161-0404	**Bromophenol Blue**, 10 g
Protein Sample Cleanup	
163-2130	**ReadyPrep 2-D Cleanup Kit**, 50 preps
163-2140	**ReadyPrep 2-D Cleanup Kit**, 5 preps
732-6221	**Micro Bio-Spin™ 6 Columns**, includes 25 columns in Tris buffer, 50 collection tubes
732-6227	**Bio-Spin® 6 Columns**, includes 25 columns in Tris buffer, 50 collection tubes
732-6228	**Bio-Spin 6 Columns**, includes 100 columns in Tris buffer, 200 collection tubes
163-2090	**ReadyPrep Reduction-Alkylation Kit**, 100 preps

Catalog #	Description
Protein Fractionation	
163-2100	**ReadyPrep Sequential Extraction Kit**, 5–15 preps
163-2085	**ReadyPrep Protein Extraction Kit (Soluble/Insoluble)**, 20 preps
163-2089	**ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear)**, 50 preps
163-2088	**ReadyPrep Protein Extraction Kit (Membrane I)**, 50 preps
163-2084	**ReadyPrep Protein Extraction Kit (Membrane II)**, 10 preps
163-2087	**ReadyPrep Protein Extraction Kit (Signal)**, 50 preps
732-6711	**Aurum™ CEX Mini Kit**, 2 preps
732-6710	**Aurum AEX Mini Kit**, 2 preps
170-2800	**MicroRotofor™ Cell Kit**, 100/120 V
170-2801	**MicroRotofor Cell Kit**, 220/240 V
170-2986	**Rotofor® Purification System**, 100/120 V
170-2987	**Rotofor Purification System**, 220/240 V
170-2926	**Model 491 Prep Cell**, 100/120 V
170-2927	**Model 491 Prep Cell**, 220/240 V
170-2908	**Mini Prep Cell without Reagent Starter Kit**
Protein Sample Depletion	
732-6701	**Aurum Serum Protein Mini Kit**, 10 preps
732-6712	**Aurum™ Affi-Gel® Blue Mini Kit**, 2 preps
163-3006	**ProteoMiner™ Protein Enrichment Small-Capacity Kit**, 10 preps for 10 mg total protein
163-3007	**ProteoMiner Protein Enrichment Large-Capacity Kit**, 10 preps for 50 mg total protein
Protein Assay Kits and Instruments	
500-0001	**Bio-Rad Protein Assay Kit I**, includes 450 ml dye reagent concentrate, bovine γ-globulin standard; sufficient for 440 standard assays or 2,200 microplate assays
500-0002	**Bio-Rad Protein Assay Kit II**, includes 450 ml dye reagent concentrate, bovine serum albumin standard; sufficient for 440 standard assays or 2,200 microplate assays
500-0111	***DC*™ Protein Assay Kit I**, includes 250 ml alkaline copper tartrate solution, 2 L dilute Folin reagent, 5 ml surfactant solution, bovine γ-globulin standard; sufficient for 450 standard assays
500-0112	***DC* Protein Assay Kit II**, includes 250 ml alkaline copper tartrate solution, 2 L dilute Folin reagent, 5 ml surfactant solution, bovine serum albumin standard; sufficient for 450 standard assays
500-0120	***RC DC*™ Protein Assay Reagents Package**, includes *RC* reagents package and *DC* reagents package, and sufficient for 450 standard assays
500-0121	***RC DC* Protein Assay Kit I**, includes *RC* reagents package, *DC* reagents package, bovine γ-globulin standard; sufficient for 450 standard assays
500-0122	***RC DC* Protein Assay Kit II**, includes *RC* reagents package, *DC* reagents package, bovine serum albumin standard; sufficient for 450 standard assays
500-0201	**Quick Start™ Bradford Protein Assay Kit 1**, includes 1× dye reagent (1 L), bovine serum albumin standard (5 × 2 mg/ml); sufficient for 200 standard assays or 4,000 microplate assays

Catalog #	Description
500-0202	**Quick Start Bradford Protein Assay Kit 2**, includes 1× dye reagent (1 L), bovine serum albumin standard set (2 sets of 7 concentration standards, 0.125–2.0 mg/ml, 2 ml)
500-0203	**Quick Start Bradford Protein Assay Kit 3**, includes 1× dye reagent (1 L), bovine γ-globulin standard (5 × 2 mg/ml)
500-0204	**Quick Start Bradford Protein Assay Kit 4**, includes 1× dye reagent (1 L), bovine γ-globulin standard set (2 sets of 7 concentration standards, 0.125–2.0 mg/ml, 2 ml)
170-2525	**SmartSpec™ Plus Spectrophotometer**
170-2502	**Standard Cuvette**, 1–3.5 ml, quartz
170-2511	**trUView™ Cuvettes**, pack of 100, individually packaged, disposable DNase- and RNase-free cuvettes

Protein Standards

161-0378	**Precision Plus Protein™ Standard Plugs**, pkg of 24, 1 mm thick agarose plugs containing 10 Strep-tagged recombinant proteins (10–250 kD), including three reference bands
161-0363	**Precision Plus Protein Unstained Standards**, 100 applications
161-0396	**Precision Plus Protein Unstained Standards Value Pack**, 500 applications
161-0373	**Precision Plus Protein All Blue Standards**, 50 applications
161-0393	**Precision Plus Protein All Blue Standards Value Pack**, 500 applications
161-0374	**Precision Plus Protein Dual Color Standards**, 50 applications
161-0394	**Precision Plus Protein Dual Color Standards Value Pack**, 250 applications
161-0375	**Precision Plus Protein™ Kaleidoscope™ Standards**, 50 applications
161-0395	**Precision Plus Protein Kaleidoscope Standards Value Pack**, 250 applications
161-0377	**Precision Plus Protein Dual Xtra Standards**, 50 applications
161-0397	**Precision Plus Protein Dual Xtra Standards Value Pack**, 250 applications
161-0385	**Precision Plus Protein™ WesternC™ Pack**, 50 applications
161-0398	**Precision Plus Protein WesternC Standards Pack Value Pack**, 250 applications
161-0399	**Precision Plus Protein WesternC Standards Value Pack**, 250 applications
161-0324	**Kaleidoscope™ Prestained Standards**, broad range, 500 µl
161-0325	**Kaleidoscope Polypeptide Standards**, 500 µl
161-0309	**Prestained SDS-PAGE Standards**, high range, 500 µl
161-0305	**Prestained SDS-PAGE Standards**, low range, 500 µl
161-0318	**Prestained SDS-PAGE Standards**, broad range, 500 µl
161-0303	**SDS-PAGE Standards**, high range, 200 µl
161-0304	**SDS-PAGE Standards**, low range, 200 µl
161-0317	**SDS-PAGE Standard**s, broad range, 200 µl
163-2093	**ReadyStrip™ 100× pH 7–10 Buffer**, includes only ampholytes, 1 ml
163-2098	**ReadyStrip 100× pH 3.9–5.1 Buffer**, includes only ampholytes, 1 ml
163-2097	**ReadyStrip 100× pH 4.7–5.9 Buffer**, includes only ampholytes, 1 ml
163-2096	**ReadyStrip 100× pH 5.5–6.7 Buffer**, includes only ampholytes, 1 ml
163-2095	**ReadyStrip 100× pH 6.3–8.3 Buffer**, includes only ampholytes, 1 ml

Bio-Lyte® Ampholyte

	pH Range							
	3/10	**3/5**	**4/6**	**6/8**	**5/7**	**5/8**	**7/9**	**8/10**
1 ml	163-2094	—	—	—	—	—	—	—
10 ml	163-1112	163-1132	163-1142	163-1162	163-1152	163-1192	163-1172	163-1182
25 ml	163-1113	—	163-1143	163-1163	163-1153	163-1193	—	—

IPG Strips and Buffers

ReadyStrip IPG strips, 12 per package.

	7 cm	**11 cm**	**17 cm**	**18 cm**	**24 cm**
pH 3–10	163-2000	163-2014	163-2007	163-2032	163-2042
pH 3–10 NL	163-2002	163-2016	163-2009	163-2033	163-2043
pH 3–6	163-2003	163-2017	163-2010	163-2035	163-2045
pH 4–7	163-2001	163-2015	163-2008	163-2034	163-2044
pH 5–8	163-2004	163-2018	163-2011	163-2036	163-2046
pH 7–10	163-2005	163-2019	163-2012	163-2037	163-2047
pH 3.9–5.1	163-2028	163-2024	163-2020	163-2038	163-2048
pH 4.7–5.9	163-2029	163-2025	163-2021	163-2039	163-2049
pH 5.5–6.7	163-2030	163-2026	163-2022	163-2040	163-2050
pH 6.3–8.3	163-2031	163-2027	163-2023	163-2041	163-2051

Catalog #	Description
Electrophoresis Instrumentation	
PROTEAN® i12™ IEF System	
164-6000	**PROTEAN i12 IEF System**, 90–240 VAC, includes basic unit, positive and negative electrode assemblies, 7 cm, 11 cm, and 17 cm focusing trays with IPG strip retainers, 1 pack each of 7 cm, 11 cm, and 17 cm rehydration/equilibration trays, 2 pairs of forceps, 2 packs electrode wicks for gel-side down and gel-side up applications, mineral oil, 2 cleaning brushes, cleaning concentrate, 2 USB flash drives, 3 styluses, pH 3–10 ReadyStrip™ IPG strips in 7 cm, 11 cm, and 17 cm lengths, rehydration sample buffer, and instruction manual. 13 cm, 18 cm, and 24 cm trays and cup loading accessories can be purchased separately
164-6001	**PROTEAN i12 IEF Cell**, 90–240 VAC basic unit includes cell, positive and negative electrode assemblies
164-6107	**7 cm i12™ Focusing Tray**, includes 2 IPG strip retainers
164-6111	**11 cm i12 Focusing Tray**, includes 2 IPG strip retainers
164-6113	**13 cm i12 Focusing Tray**, includes 2 IPG strip retainers
164-6117	**17 cm i12 Focusing Tray**, includes 2 IPG strip retainers
164-6118	**18 cm i12 Focusing Tray**, includes 2 IPG strip retainers
164-6124	**24 cm i12 Focusing Tray**, includes 2 IPG strip retainers
165-4035	**7 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
165-4025	**11 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
164-6313	**13 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
165-4015	**17 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
165-4041	**18 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
165-4043	**24 cm i12 Rehydration/Equilibration Tray**, with lids, pkg of 25
164-6040	**IPG Strip Retainers**, pkg of 2
164-6020	**i12 Sample Cup Holder**, pkg of 1, 12-position sample cup holder, includes 25 disposable sample cups
164-6021	**i12 Sample Cups**, pkg of 25
164-6030	**Gel-Side Up Electrode Wicks**, pkg of 100
164-6031	**Gel-Side Down Electrode Wicks**, pkg of 500
164-6012	**Negative Electrode Assembly**, pkg of 1
164-6011	**Positive Electrode Assembly**, pkg of 1
164-6010	**Electrode Assembly Pair**, pkg of 1 pair, positive and one negative electrode assemblies
165-4072	**Cleaning Brushes**, pkg of 2
161-0722	**Cleaning Concentrate**
164-6060	**USB Flash Drive**, pkg of 2
164-6050	**Stylus**, pkg of 3
165-4070	**Forceps**, pkg of 1
163-2129	**Mineral Oil**
163-2105	**ReadyPrep 2-D Starter Kit**

Catalog #	Description
Mini-PROTEAN® Tetra Cells and Systems	
165-8000	**Mini-PROTEAN Tetra Cell**, 10-well, 0.75 mm thickness; 4-gel system includes 5 combs, 5 sets of glass plates, 2 casting stands, 4 casting frames, sample loading guide, electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam
165-8001	**Mini-PROTEAN Tetra Cell**, 10-well, 1.0 mm thickness; 4-gel system includes 5 combs, 5 sets of glass plates, 2 casting stands, 4 casting frames, sample loading guide, electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam
165-8002	**Mini-PROTEAN Tetra Cell**, 10-well, 0.75 mm thickness; 2-gel system includes 5 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, electrode assembly, tank, lid with power cables, mini cell buffer dam
165-8003	**Mini-PROTEAN Tetra Cell**, 10-well, 1.0 mm thickness; 2-gel system includes 5 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, electrode assembly, tank, lid with power cables, mini cell buffer dam
165-8004	**Mini-PROTEAN Tetra Cell for Mini Precast Gels**, 4-gel system includes electrode assembly, clamping frame, companion module, tank, lid with power cables, mini cell buffer dam
165-8005	**Mini-PROTEAN Tetra Cell for Mini Precast Gels**, 2-gel system includes electrode assembly, clamping frame, tank, lid with power cables, mini cell buffer dam
165-8006	**Mini-PROTEAN Tetra Cell**, 10-well, 1.5 mm thickness; 4-gel system includes 5 combs, 5 sets of glass plates, 2 casting stands, 4 casting frames, sample loading guide, electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam
165-8007	**Mini-PROTEAN Tetra Cell**, 10-well, 1.5 mm thickness; 2-gel system includes 5 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, electrode assembly, tank, lid with power cables, mini cell buffer dam
165-8025	**Mini-PROTEAN Tetra Cell and PowerPac™ Basic Power Supply**, includes 165-8001 and 164-5050
165-8026	**Mini-PROTEAN Tetra Cell and PowerPac™ Universal Power Supply**, includes 165-8001 and 164-5070
165-8027	**Mini-PROTEAN Tetra Cell and PowerPac™ HC Power Supply**, includes 165-8001 and 164-5052
165-8028	**Mini-PROTEAN Tetra Cell and PowerPac™ HV Power Supply**, includes 165-8001 and 164-5056
165-8029	**Mini-PROTEAN Tetra Cell and Mini Trans-Blot® Module**, includes 165-8001 and 170-3935
165-8030	**Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels and Mini Trans-Blot Module**, includes 165-8004 and 170-3935
165-8033	**Mini-PROTEAN Tetra Cell, Mini Trans-Blot Module, and PowerPac Basic Power Supply**, includes 165-8001, 170-3935, and 164-5050

Catalog #	Description
Mini-PROTEAN® Tetra Cells and Systems (contd.)	
165-8034	**Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels, Mini Trans-Blot Module, and PowerPac Basic Power Supply**, includes 165-8004, 170-3935, and 164-5050
165-8035	**Mini-PROTEAN Tetra Cell, Mini Trans-Blot Module, and PowerPac HC Power Supply**, includes 165-8001, 170-3935, and 164-5052
165-8036	**Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels, Mini Trans-Blot Module, and PowerPac HC Power Supply**, includes 165-8004, 170-3935, and 164-5052
Mini-PROTEAN Dodeca Cells and Systems	
165-4100	**Mini-PROTEAN® 3 Dodeca™ Cell**, includes electrophoresis tank with built-in cooling coil, lid with power cables, 6 electrophoresis clamping frames, 2 buffer dams, drain line, 2 gel releasers
165-4101	**Mini-PROTEAN 3 Dodeca Cell with Multi-Casting Chamber**, same as 165-4100 with multi-casting chamber, 15 separation sheets, 8 acrylic blocks, tapered luer connector, stopcock valve
Criterion™ Cells and Systems	
165-6001	**Criterion Cell**, includes buffer tank, lid with power cables, 3 sample loading guides (12 + 2-well, 18-well, 26-well)
165-6019	**Criterion Cell and PowerPac Basic Power Supply**, 100–120/220–240 V, includes 165-6001 and 164-5050
Criterion™ Dodeca™ Cells and Systems	
165-4130	**Criterion Dodeca Cell**, includes electrophoresis buffer tank with built-in cooling coil, lid with power cables
165-4138	**Criterion Dodeca Cell and PowerPac HC Power Supply**, includes 165-4130 and 164-5052
165-4139	**Criterion Dodeca Cell and PowerPac Universal Power Supply**, includes 165-4130 and 164-5070
165-5133	**Criterion Dodeca Cell and 6-Row AnyGel™ Stand**, includes 165-4130 and 165-5131
PROTEAN® II xi Cells	
165-1801	**PROTEAN II xi Cell**, 16 cm, without spacers and combs
165-1802	**PROTEAN II xi Cell**, 16 cm, 1.5 mm spacers (4), 15-well combs (2)
165-1803	**PROTEAN II xi Cell**, 16 cm, 1.0 mm spacers (4), 15-well combs (2)
165-1804	**PROTEAN II xi Cell**, 16 cm, 0.75 mm spacers (4), 15-well combs (2)
165-1811	**PROTEAN II xi Cell**, 20 cm, without spacers and combs
165-1812	**PROTEAN II xi Cell**, 20 cm, 1.5 mm spacers (4), 15-well combs (2)
165-1813	**PROTEAN II xi Cell**, 20 cm, 1.0 mm spacers (4), 15-well combs (2)
165-1814	**PROTEAN II xi Cell**, 20 cm, 0.75 mm spacers (4), 15-well combs (2)
PROTEAN II XL Cells	
165-3188	**PROTEAN II XL Cell**, wide format 1-D vertical electrophoresis cell, 1.0 mm, includes PROTEAN II xi basic unit (#165-1834) and 1.0 mm IPG conversion kit (#165-3183)
165-3189	**PROTEAN II XL Cell**, wide format 1-D vertical electrophoresis cell, 1.5 mm, includes PROTEAN II xi basic unit (#165-1834) and 1.5 mm IPG conversion kit (#165-3186)
165-3190	**PROTEAN II XL Cell**, wide format 1-D vertical electrophoresis cell, 2.0 mm, includes PROTEAN II xi basic unit (#165-1834) and 2.0 mm IPG conversion kit (#165-3184)
165-1815	**PROTEAN II xi Cell 2-D Conversion Kit**, converts PROTEAN II xi cell into a tube gel IEF 2-D system; includes 2 tube gel adaptors, 24 glass tubes (1.5 mm ID, 180 mm length), gaskets, grommets, stoppers
165-3183	**PROTEAN II xi Cell IPG Conversion Kit**, 1.0 mm, 18.5 × 20 cm, for conversion to IPG PROTEAN II XL system; includes IPG clamps, 20 × 20 cm glass plates (2), IPG spacers, 2-D combs, and central cooling core gaskets
165-3186	**PROTEAN II xi Cell IPG Conversion Kit**, 1.5 mm, 18.5 × 20 cm, for conversion to IPG PROTEAN II XL system; includes IPG clamps, 20 × 20 cm glass plates (2), IPG spacers, 2-D combs, and central cooling core gaskets
165-3184	**PROTEAN II xi Cell IPG Conversion Kit**, 2.0 mm, 18.5 × 20 cm, for conversion to IPG PROTEAN II XL system; includes IPG clamps, 20 × 20 cm glass plates (2), IPG spacers, 2-D combs, and central cooling core gaskets
165-1834	**PROTEAN II xi Basic Unit With Casting Stand**, vertical electrophoresis system, includes electrophoresis cell with central cooling core, gel casting stand
165-1951	**PROTEAN II xi Multi-Cell**, multi-cell electrophoresis system, includes 3 central cooling cores, buffer tank, PROTEAN II xi multi-casting chamber with accessories
165-1956	**PROTEAN II xi Multi-Cell 2-D Conversion Kit**, for proper cooling in 2-D electrophoresis applications; includes 2 cooling coils and manifold
165-3176	**PROTEAN II XL Multi-Cell**, wide format vertical electrophoresis multi-cell, 1.0 mm, compatible with ReadyStrip IPG strips; includes catalog #165-1951, #165-1956, and 3 PROTEAN II xi cell IPG conversion kits of desired thickness
165-3177	**PROTEAN II XL Multi-Cell**, wide format vertical electrophoresis multi-cell, 1.5 mm, compatible with ReadyStrip IPG strips; includes catalog #165-1951, #165-1956, and 3 PROTEAN II xi cell IPG conversion kits of desired thickness
165-3178	**PROTEAN II XL Multi-Cell**, wide format vertical electrophoresis multi-cell, 2.0 mm, compatible with ReadyStrip IPG strips; includes catalog #165-1951, #165-1956, and 3 PROTEAN II xi cell IPG conversion kits of desired thickness
PROTEAN® Plus Dodeca™ Cells and Systems	
165-4150	**PROTEAN Plus Dodeca Cell**, 100/120 V, includes electrophoresis buffer tank with built-in ceramic cooling core, lid, buffer recirculation pump with tubing, 2 gel releasers
165-4140	**PROTEAN Plus Dodeca Cell (100/120 V) and PowerPac HC Power Supply**, includes 165-4150 and 164-5052
165-4142	**PROTEAN Plus Dodeca Cell (100/120 V) and PowerPac Universal Power Supply**, includes 165-4150 and 164-5070
165-4144	**PROTEAN Plus Dodeca Cell (100/120 V), Trans-Blot Plus Cell, and PowerPac Universal Power Supply**, includes 165-4150, 170-3990, and 164-5070

Catalog #	Description
PROTEAN® Plus Dodeca™ Cells and Systems (contd.)	
165-5134	**PROTEAN Plus Dodeca Cell (100/120 V) and Two 6-Row AnyGel Stands**, includes 165-4150 and two 165-5131
165-4151	**PROTEAN Plus Dodeca Cell**, 220/240 V, includes electrophoresis buffer tank with built-in ceramic cooling core, lid, buffer recirculation pump with tubing, 2 gel releasers
165-4141	**PROTEAN Plus Dodeca Cell (220/240 V) and PowerPac HC Power Supply**, includes 165-4151 and 164-5052
165-4143	**PROTEAN Plus Dodeca Cell (220/240 V) and PowerPac Universal Power Supply**, includes 165-4151 and 164-5070
165-4145	**PROTEAN Plus Dodeca Cell (220/240 V) Trans-Blot Plus Cell, and PowerPac Universal Power Supply**, includes 165-4151, 170-3990, and 164-5070
165-5135	**PROTEAN Plus Dodeca Cell (220/240 V) and Two 6-Row AnyGel Stands**, includes 165-4151 and two 165-5131
Power Supplies	
164-5050	**PowerPac™ Basic Power Supply**, 100–120/220–240 V
164-5052	**PowerPac HC Power Supply**, 100–120/220–240 V
164-5056	**PowerPac HV Power Supply**, 100–120/220–240 V
164-5070	**PowerPac Universal Power Supply**, 100–120/220–240 V
IEF and SDS-PAGE Buffers and Reagents	
163-2111	**ReadyPrep Overlay Agarose**, 1 bottle, 50 ml
161-0732	**10× Tris/Glycine/SDS**, 1 L
161-0734	**10× Tris/Glycine**, 1 L
161-0744	**10× Tris/Tricine/SDS**, 1 L
161-0788	**XT MOPS Running Buffer**, 20×, 500 ml
161-0789	**XT MES Running Buffer**, 20×, 500 ml
161-0790	**XT Tricine Running Buffer**, 20×, 500 ml
161-0793	**XT MOPS Buffer Kit**, includes 500 ml 20× XT MOPS running buffer, 10 ml 4× XT sample buffer, 1 ml 20× XT reducing agent
161-0796	**XT MES Buffer Kit**, includes 500 ml 20× XT MES running buffer, 10 ml 4× XT sample buffer, 1 ml 20× XT reducing agent
161-0797	**XT Tricine Buffer Kit**, includes 500 ml 20× XT Tricine running buffer, 10 ml 4× XT sample buffer, 1 ml 20× XT reducing agent
161-0729	**EDTA**, 500 g
161-0718	**Glycine**, 1 kg
161-0713	**Tricine**, 500 g
161-0719	**Tris**, 1 kg
161-0404	**Bromophenol Blue**, 10 g
Precast Gels*	
Mini-PROTEAN® TGX™ Precast Gels (for 7 cm IPG Strips) IPG well, 10 gels per box	
456-1021	**7.5% Mini-PROTEAN TGX Precast Gel**
456-1031	**10% Mini-PROTEAN TGX Precast Gel**
456-1041	**12% Mini-PROTEAN TGX Precast Gel**
456-1081	**4–15% Mini-PROTEAN TGX Precast Gel**
456-1091	**4–20% Mini-PROTEAN TGX Precast Gel**
456-9031	**Any kD™ Mini-PROTEAN TGX Precast Gel**

Catalog #	Description
Mini-PROTEAN® TGX Stain-Free™ Precast Gels (for 7 cm IPG Strips)	
456-8021	**7.5% Mini-PROTEAN TGX Stain-Free Precast Gel**
456-8031	**10% Mini-PROTEAN TGX Stain-Free Precast Gel**
456-8041	**12% Mini-PROTEAN TGX Stain-Free Precast Gel**
456-8121	**Any kD Mini-PROTEAN TGX Stain-Free Precast Gel**
Criterion Precast Gels (for 11 cm IPG Strips) IPG +1 well, package of 1	
345-0101	**10% Criterion Tris-HCl Precast Gel**
345-0102	**12.5% Criterion Tris-HCl Precast Gel**
345-0103	**4–15% Criterion Tris-HCl Precast Gel**
345-0104	**4–20% Criterion Tris-HCl Precast Gel**
345-0105	**8–16% Criterion Tris-HCl Precast Gel**
345-0106	**10.5–14% Criterion Tris-HCl Precast Gel**
345-0107	**10–20% Criterion Tris-HCl Precast Gel**
345-0115	**10% Criterion XT Bis-Tris Precast Gel**
345-0121	**12% Criterion XT Bis-Tris Precast Gel**
345-0127	**4–12% Criterion XT Bis-Tris Precast Gel**
345-0133	**3–8% Criterion XT Tris-Acetate Precast Gel**
345-8161	**8–16% Criterion Stain-Free Precast Gel**
Criterion™ TGX™ Precast Gels (for 11 cm IPG Strips)	
567-1071	**18% Criterion TGX Precast Gel**
567-1081	**4–15% Criterion TGX Precast Gel**
567-1091	**4–20% Criterion TGX Precast Gel**
567-1101	**8–16% Criterion TGX Precast Gel**
567-1111	**10–20% Criterion TGX Precast Gel**
567-1121	**Any kD Criterion TGX Precast Gel**
Criterion™ TGX Stain-Free™ Precast Gels (for 11 cm IPG Strips)	
567-8071	**18% Criterion TGX Stain-Free Precast Gel**
567-8081	**4–15% Criterion TGX Stain-Free Precast Gel**
567-8091	**4–20% Criterion TGX Stain-Free Precast Gel**
567-8101	**8–16% Criterion TGX Stain-Free Precast Gel**
567-8111	**10–20% Criterion TGX Stain-Free Precast Gel**
567-8121	**Any kD Criterion TGX Stain-Free Precast Gel**

* For a complete selection of precast gels, visit www.bio-rad.com

Gel Casting Buffers and Reagents

161-5100	**SDS-PAGE Reagent Starter Kit**, includes 100 g acrylamide, 5 g bis, 5 ml TEMED, 10 g ammonium persulfate
161-0100	**Acrylamide**, 99.9%, 100 g
161-0120	**Acrylamide/Bis Powder**, 19:1, 30 g
161-0122	**Acrylamide/Bis Powder**, 37.5:1, 30 g
161-0140	**40% Acrylamide Solution**, 500 ml
161-0144	**40% Acrylamide/Bis Solution**, 19:1, 500 ml
161-0146	**40% Acrylamide/Bis Solution**, 29:1, 500 ml
161-0148	**40% Acrylamide/Bis Solution**, 37.5:1, 500 ml
161-0154	**30% Acrylamide/Bis Solution**, 19:1, 500 ml
161-0156	**30% Acrylamide/Bis Solution**, 29:1, 500 ml
161-0158	**30% Acrylamide/Bis Solution**, 37.5:1, 500 ml
161-0200	**Bis Crosslinker**, 5 g

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Catalog #	Description

Gel Casting Buffers and Reagents (contd.)

161-0800	**TEMED**, 5 ml
161-0798	**Resolving Gel Buffer**, 1.5 M Tris-HCl, pH 8.8, 1 L
161-0700	**Ammonium Persulfate (APS)**, 10 g
161-0799	**Stacking Gel Buffer**, 0.5 M Tris-HCl, pH 6.8, 1 L

Gel Casting Accessories

See catalog or **www.bio-rad.com** for a complete listing of accessories, including available empty gel cassettes and glass plates, spacers, combs, etc.

165-5131	**AnyGel™ Stand**, 6-row, holds 6 PROTEAN gels, 12 Criterion gels, or 18 Ready Gel mini gels
165-4131	**AnyGel Stand**, single-row, holds 1 PROTEAN gel, 2 Criterion gels, or 3 Ready Gel mini gels
165-4122	**Model 485 Gradient Former and Mini-PROTEAN 3 Multi-Casting Chamber**, includes 165-4120 and 165-4110
165-4123	**Model 495 Gradient Former and PROTEAN Plus Multi-Casting Chamber**, includes 165-4121 and 165-4160

Total Protein Gel Stains

161-0786	**Bio-Safe™ Coomassie Stain**, 1 L
161-0787	**Bio-Safe Coomassie Stain**, 5 L
161-0449	**Silver Stain Plus™ Kit**, includes fixative enhancer concentrate, silver complex solution, reduction moderator solution, image development reagent, development accelerator reagent, stains 13 full size or 40 mini gels
161-0496	**Oriole™ Fluorescent Gel Stain**, 1× solution, 1 L
161-0492	**Flamingo™ Fluorescent Gel Stain**, 10× solution, 500 ml
170-3125	**SYPRO Ruby Protein Gel Stain**, 1× solution, 1 L
161-0440	**Zinc Stain and Destain Kit**, includes 125 ml of 10× zinc stain solution A, 125 ml of 10× zinc stain solution B, 125 ml of 10× zinc destain solution
161-0470	**Copper Stain and Destain Kit**, includes 125 ml of 10× copper stain, 125 ml of 10× copper destain solution

Catalog #	Description

High-Throughput Stainers

165-3400	**Dodeca Stainer**, large, 100–240 V, includes 13 trays (12 clear, 1 white), 12 tray attachments, shaking rack, solution tank, lid with shaker motor, shaker control unit, gel clip
165-3401	**Dodeca Stainer**, small, 100–240 V, includes 13 trays (12 clear, 1 white), 12 Criterion tray attachments, shaking rack, solution tank, lid with shaker motor, shaker control unit, gel clip

Imaging Systems and Spot Cutter

170-7991	**GS-900™ Calibrated Densitometry System**, gel densitometry system, PC compatible, scanner, cables, Image Lab software, optional 21 CFR Part 11 and Instrument Qualification/Operations Qualification
170-8280	**ChemiDoc™ MP System**, gel imaging system, PC or Mac, includes darkroom, UV transilluminator, epi-white illumination, camera, power supply, cables, Image Lab™ software
170-8270	**Gel Doc EZ System**, gel imaging system, PC or Mac, includes darkroom, camera, cables, Image Lab software; samples trays (#170-8271, 170-8272, 170-8273, or 170-8274) are sold separately; sample trays are required to use the system
170-9460	**Molecular Imager® PharosFX™ Plus System**, PC or Mac, 110–240 V, includes Quantity One® software, sample tray set, fluorescence filters (170-7866, 170-7896) and phosphor imaging filters, USB2 cable
170-9450	**Molecular Imager PharosFX System**, PC or Mac, 110–240 V, includes Quantity One software, sample tray set, fluorescence filters (170-7866, 170-7896), USB2 cable
165-7200	**EXQuest™ Spot Cutter**, gel excision instrument, includes enclosure, imaging system, fluidics system, robotics, sensors, cutting head, gel tray, microplate rack, wash station
165-7201	**EXQuest Spot Cutter with PC**, gel excision instrument, includes PC, enclosure, imaging system, fluidics system, robotics, sensors, cutting head, gel tray, microplate rack, wash station

Analysis Software

170-9690	**Image Lab Software**
170-9600	**Quantity One 1-D Analysis Software**, PC or Mac
170-9630	**PDQuest™ Advanced 2-D Analysis Software**

Gel Drying Supplies

165-1771	**GelAir™ Drying System**, 115 V, 60 Hz, includes 165-1777, 2 drying frames, 16 clamps, assembly table, 50 precut sheets of cellophane support, gel drying solution
165-1777	**GelAir Dryer**, 115 V, 60 Hz, gel drying oven only

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